MODULATION OF VASCULAR TONE BY ACTIVATORS AND INHIBITORS OF MEMBRANE POTASSIUM CHANNELS IN ISOLATED CONDUCTANCE AND CAPACITANCE BLOOD VESSELS WITH ESPECIAL REGARD TO LEVOSIMENDAN, AN INODILATOR DRUG

Ph.D. Thesis

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Ph.D. Thesis

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I.

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SUMMARY

The main findings of the present thesis are as follows:

1. ATP-sensitive (K_{ATP}) and large conductance calcium-activated (BK_{Ca}) potassium channels are effectors in modulating the smooth muscle tone in canine coronary artery. These ion channels are functionally two distinct targets for activators and inhibitors of potassium channels. Nitric oxide activates BK_{Ca} channels both in the conduit epicardial coronary artery of the dog and in the capacitance vein isolated from human subjects. This novel regulatory role of nitric oxide in the vascular smooth muscle may have a protective role against pathological vasoconstriction through hyperpolarization of the cell membrane.

2. Voltage dependent potassium (K_V) and BK_{Ca} channels regulate the basal (resting) tone in human, porcine and canine epicardial coronary arteries, respectively. Coronary artery samples isolated from the porcine heart can serve as models for studying the functional effect of drugs on K_V channels and canine coronary arteries for studying those acting on BK_{Ca} channels.

3. Apart from endothelial nitric oxide in conduit arteries, the adventitial neurotransmission is also able to modulate the tone of vascular smooth muscle in capacitance veins. In canine saphenous vein K_V channels regulate noradrenergic neurotransmission and profoundly influence the passive (basal) tension of the smooth muscle.

4. Vasorelaxing mechanism of the inodilator, levosimendan, involves the activation of K_V, and, at large concentrations of BK_{Ca} channels in porcine isolated epicardial coronary artery. The mechanism of vasodilation induced by levosimendan depends, at least in part, on two other potassium channels functionally differing from the known effect of the drug on K_{ATP} channels.

5. The K_{ATP} channel activator, cromakalim is able to relax human isolated portal vein. Levosimendan is found to be more potent than cromakalim and its mechanism partially depends on activation of hyperpolarizing K_{ATP} channels.

6. Levosimendan dilates the human saphenous vein preparations by interacting with hyperpolarizing potassium channels, K_{ATP} and BK_{Ca}. The K_{ATP} channels partly mediate the

venodilating effect of levosimendan and the presence of intact BK_{Ca} channels are obligatory for the dilating effect of the inodilator.

7. Vasodilating capacity of the inodilator, levosimendan, depends on the actual value of transmural pressure in both the bypass conduit and human internal mammary arteries, furthermore in the capacitance canine saphenous vein. In this 'myogenic response' hyperpolarizing potassium channels, probably K_{ATP}-types, may play some roles.

ABBREVIATIONS

4-AP	= 4-aminopyridine
5-HT	= 5-hydroxytryptamine
6-OHDA	= 6-hydroxydopamine
Ach	= acetylcholine
BK_{Ca}	= large conductance calcium activated potassium channel
BRK	= bradykinin
ChTX	= charybdotoxin
CRO	= cromakalim
cGMP	= cyclic guanosine 3,5-monophosphate
EDHF	= endothelium derived hyperpolarizing factor
FDT	= force-displacement transducer (Type F30)
GLI	= glibenclamide
IBTX	= iberiotoxin
INDO	= indomethacin
K_{ATP}	= ATP-sensitive potassium channel
K_{Ca}	= calcium-dependent potassium channel
KCl	= potassium chloride
KHS	= Krebs-Henseleit solution
K_{IR}	= inwardly rectifying potassium channel
K_V	= voltage-dependent potassium channel
LEVO	= levosimendan
L-NOARG	= NG-nitro-L-arginine
L-VOCC	= L-type voltage dependent potassium channel
NA	= noradrenaline
NIS	= nisoldipine
NO	= nitric oxide
NTG	= nitroglycerine
$PGF_{2\alpha}$	= prostaglandin $F_{2\alpha}$
PN	= pen recorder (Type 175, KUTESZ)
TEA	= tetraethylammonium

CONTENTS

1. INTRODUCTION

Cardiovascular diseases are still at the first place of morbidity and mortality in the Western societies. A new class of substances affecting the membrane potential of the heart and the circulatory tree are of current interest. Possible targets of these natural or synthetic compounds are hyperpolarizing potassium channels located on the surface of the smooth muscle membrane. In the vasculature a large background potassium current renders the smooth muscle in a resting, hyperpolarized state. This ion current inhibits the entry of extracellular calcium ions into the intracellular space of the arterial and venous smooth muscle. Since calcium ion flow through the voltage-operated calcium channels is a common mechanism of elevated tone in the blood vessels, activation of potassium channels would be a therapeutic approach for inducing vasodilation independently of the underlying pathological insult.

Potassium channels by hyperpolarizing the smooth muscle membrane regulate both the arterial and venous tones. Activators and inhibitors of potassium channels may have therapeutic values in several cardiovascular disorders, including hypertension, atherosclerosis, heart failure, diabetes, and some types of angina pectoris with dynamic stenosis. Potassium channels are the most diverse ion channels and their localizations and functions depend on the animal species and types of blood vessels. Therefore, investigations of these channels on human blood vessels are essential.

There are three main types of potassium channels in the vascular system: the ATP-sensitive potassium channel (K_{ATP}) in the family of inwardly rectifying potassium channels (K_{IR}), the voltage dependent (K_V) and the calcium activated potassium channels (K_{Ca}). In our experiments we focused our attention on the regulatory roles of these potassium channels exerted on the tone of some blood vessels including human ones. Specific activators and inhibitors of potassium channels as well as the possible involvement of the function of potassium channels in the vasodilating mechanism of nitric oxide and that of the new inodilator drug, levosimendan, were investigated by using isolated conduit arteries and capacitance veins.

1.1. Anatomical summary of blood vessels

Arteries and veins are muscular blood vessels and their walls are composed of three layers: tunica intima, media and adventitia. On the border between the media and the intima

as well as the media and adventitia there are sheets of elastic fibers. They show a common general pattern of organization and are made up of similar tissue materials (Basar & Weiss 1981, Rhodin 1980). The intima consists of a single layer of endothelial cells which is in contact with the blood and a thin subendothelial layer with fibroblasts and collagen fibers. The endothelium interacts with smooth muscle cells by the synthesis and secretion of vasoactive mediators or by direct contact via gap junctions.

The media of large central arteries including the conduit type of side branches is built up of smooth muscle cells and connective tissue, elastic and collagen fibers. The outermost layer is the adventitia, which may, in some places, be as thick as the media and it is composed of loose connective tissue containing relatively sparse elastin and collagen fibers running in a predominantly longitudinal direction. The adventitial layer carries the majority of sympathetic nerves. The density of innervations varies widely and probably reflects the contribution of the individual vessels to centrally controlled responses.

1.2. Functional differentiation of vascular bed

Coordination and integration of cardiac and peripheral vascular activity are essential for the maintenance of homeostasis in the organism. The peripheral circulation plays an essential role in this interplay and can be classified in different categories: conduit, capacitance and resistance blood vessels (Basar & Weiss 1981, Safar 1996, Witzleb 1989).

1.2.1. The arteries, as conduits, have the role of carrying an adequate supply of blood from the heart to the peripheral organs and tissues of the body. The efficiency of the arterial conduit function depends on the arterial caliber and the constancy of mean blood pressure.

1.2.2. Capacitance vessels (mainly the venous section) can take in or pass on large volumes of blood with no marked effects on the other parameters of the circulation. They can thus act as blood reservoirs. Changes of smooth muscle tone in these vessels can produce haemodinamically important shifts in regional blood content and thus influence venous return and cardiac output.

1.2.3. Resistance vessels determine the overall resistance function, and hence, blood flow. The greatest resistance to flow is in the precapillary region (terminal arteries and arterioles). Activity of the smooth muscles in these arteries is the decisive factor in the regulation of volume flow within each vascular bed, as well as in the distribution of the cardiac output among the various organs. The postcapillary resistance is determined by the venules (and veins).

1.3. Blood vessel regulation via vasoactive agents

The smooth muscle tone is regulated by different vasoconstrictor or vasodilator agents regulating the diameter of blood vessel and hence can induce haemodynamic changes in the perfusion of the tissue. Important agents playing a role in the regulation of blood vessels involved in this study are the following:

5-hydroxytryptamine(5-HT): 5-HT has complex effect on the cardiovascular system, including direct and indirect vasoconstriction or vasodilation (Martin 1994). 5-HT is a potent vasoconstrictor agent in coronary arteries and has been used in the provocation of coronary vasospasm (Perez et al 1983). Moreover, 5-HT can be taken up by adrenergic nerve endings of some blood vessels and can be released from them together with NA (Szabó et al 1991).

Nitric oxide (NO): The endothelium-derived relaxing factor, which was firstly characterized by Furchgott and Zawadzki in 1980, and identified as NO (Furchgott 1988, Ignarro et al 1988), induces smooth muscle relaxation due to the activation of guanylyl cyclase. The complex mechanisms involved in the synthesis and effect of NO is well established (Pearson & Vanhoutte, 1993). Moreover, NO, besides adenosine, participates in coronary artery autoregulation (Ishibashi et al 1998).

Endothelium-dependent hyperpolarizing factor (EDHF): It has been demonstrated that endothelium-dependent relaxation induced by acetylcholine (Ach) and bradykinin (BRK) persists in the presence of cyclo-oxygenase or NO synthase inhibitors (Prieto et al 1998, Krassói et al 2000) and called endothelium-dependent hyperpolarizing factor. This is an important mediator of vascular relaxation in different vascular beds, however, the chemical or physical nature of EDHF is unknown (Campbell & Gauthier 2002).

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$): This metabolite of arachidonic acid is a powerful contractile mediator in the coronary circulation.

Noradrenaline (NA): The sympathetic neurotransmitter in most vessels activates postjunctional α-adrenoreceptors initiating contraction of the vascular smooth muscle cells and thus vasoconstriction. However in several vascular beds, e.g. coronary arteries, activation of postjunctional β-adrenergic receptors by liberated NA induces vasodilation under physiological conditions (Vanhoutte et al 1981).

Potassium and calcium ion channels (K and Ca channels): In addition to stimulation of receptors located on the cell membrane, the other main cellular mechanism by which vascular smooth muscles can be activated is membrane depolarization. Opening of potassium channels leads to diffusion of potassium ions out of the cells, causes membrane

hyperpolarization and decreases the activation of voltage-gated Ca^{2+} channels. The decrease in intracellular Ca^{2+} concentration results in vasodilation (Jackson 2000).

1.4. Role of potassium channels in the modulation of smooth muscle cells

Smooth muscle cells are the most intensively investigated vascular cells. Membrane potential of smooth muscle cells appears to be an important regulator of vascular tone. Vascular smooth muscle cells express 4 different types of potassium channels: K_V, BK_{Ca}, K_{ATP} and G-protein coupled inward rectifier potassium (GIRK) channels (Nelson & Quayle 1995, Hobbs et al 2004). The opening of potassium channels in the membrane of smooth muscle cells increases potassium efflux out of the cells leading to membrane hyperpolarization. Closure of potassium channels has the opposite effect. Hyperpolarization closes the voltage-gated Ca^{2+} channels, which causes vasodilation, whereas depolarization opens them inducing vasoconstriction (Jackson 2000). K_V channels have been identified in smooth muscle cells obtained from different types of blood vessels. 4-AP is the known most selective inhibitor of K_V channels in vascular smooth muscle. Inhibition of the 4-AP-sensitive potassium channels induces depolarization and vasoconstriction (Nelson & Quayle 1995, Halliday et al. 1995, Knot & Nelson 1995, Nelson & Brayden 1993). The K_{Ca} channels are found in smooth muscle cells obtained from different types of vascular beds. These channels are activated by an increase in intracellular Ca^{2+}. Blockade of these channels by tetraethylammonium (TEA) leads to membrane depolarization and vasoconstriction (Nelson & Quayle 1995; Silva et al 1994; Cook 1989). The role of K_{ATP} channels in the regulation of membrane potential is controversial. Glibenclamide (GLI), the selective blocker of K_{ATP} channels, caused arteriolar constriction in several microcirculatory beds, but in other blood vessels its effect is not obvious under physiological conditions. The channel seems to be involved in the metabolic regulation of blood flow and is activated in pathological conditions, such as hypoxia (Nelson & Quayle 1995). The role of K_{IR} channels in the regulation of resting membrane potential and tone remains unclear. The K_{IR} channel in arterial smooth muscle is very sensitive to inhibition by extracellular Ca^{2+}. Recent evidences suggest that potassium-induced vasodilation in resistance arteries is mediated by K_{IR} channels but this channel does not play a functional role in the regulation of tone in conduit blood vessels (Nelson & Quayle 1995).

1.5. Role of potassium channels in the vasodilating effect of endothelial nitric oxide

The vascular endothelium is located at the interface between the circulating blood and vessel wall. Endothelial cells, unlike nerve and smooth muscle cells, are classified as nonexcitable cells since they have never been observed to produce action potentials. The endothelium interacts with smooth muscle cells by the synthesis and secretion of vasoactive mediators including nitric oxide and, possibly, EDHF/s. Although in most vascular beds, EDHF is considered to be a sole entity, it was suggested that endothelial NO is also able to hyperpolarize the smooth muscle (Tare et al 1990). NO is known to activate Ca^{2+}-activated potassium channels on the membrane of the smooth muscle which may differ from those potassium channels that are located on the endothelial membrane (Prieto et al 1998; Nilius et al 1997; Simonsen et al 1995).

1.6. Role of potassium channels in the regulation of adrenergic nerves

Blood vessels are innervated by sympathetic nerves. NA, released from the sympathetic nerve terminals, is the main transmitter in the adrenergic nervous system. NA activates postjunctional α-adrenoreceptors, which initiates the contraction of vascular smooth muscle. The release of this transmitter from presynaptic nerve terminal depends on the electrical responses of presynaptic membrane. Potassium channels are important participants of electrical changes in membrane potential leading to transmitter release (Vanhoutte et al 1981).

Similarly to the arteries, the tone of the veins is also controlled by the sympathetic nervous system. K_V channels located on the perivascular nerves were suggested to regulate the tone of the venous smooth muscle indirectly by releasing NA (Kato & Takata 1987). K_{Ca} channels were also proved to regulate NA release from the perivascular nerves of arteries (Marin et al 1985; Nally & Muir 1992), however, the functional roles of autonomic neuronal K_{IR} channels including the K_{ATP} subtype could not be detected (Deist et al 1992; Lee et al 1995; Roeper & Pongs 1996; Ponce et al 1996).

1.7. Effect of simulated transmural pressure on the activation of potassium channels

Acute changes in transmural pressure of blood vessels can influence the activity of potassium channels and thus the effect of potassium channel openers. In an in vitro organ

bath, passive stretch applied perpendicular to the axis of an artery or vein represents the increase of transmural pressure being physiological at 80-100 mmHg in a large conduit artery and 10 mmHg or lower in a capacitance vein. An increase in transmural pressure causes depolarization and under these conditions, opening of K_{ATP} channels can induce a much larger change in membrane potential than in relaxed arteries (Daut et al 1994). Stretching of isolated veins also induces an acute myogenic response and changes the membrane potential (Bérczi et al 1992; Monos et al 1993). Other mechanosensitive ion channels known to involve in stretch-responses are the following: non-selective cation channels, K_{Ca} channels and tandem-pore potassium channels (Wu et al 2003; Lee et al 2000; Bang et al 2000).

1.8. Aims of the present study

On the basis of these findings, BK_{Ca}, K_V and K_{ATP} channels seem to be important potassium channels in the regulation of the tone of smooth muscle cells. The main purpose of the present study was to determine the functional role of K_{ATP}, BK_{Ca} and K_V channels using some potassium channel blockers and as well as known putative potassium channel activators.

The aims were subdivided as follows:

- Investigation of the function of potassium channels in conduit and capacitance types of blood vessels by using activators and inhibitors.
- Investigation of the role of BK_{Ca} channels in the vasodilating effect of NO.
- Investigation of the role of K_V channels in the regulation of NA release.
- Investigation of the role of K_{ATP} and BK_{Ca} channels on the venous tone.
- Characterization of potassium channels involved in the vasodilating mechanism of the inodilator, LEVO, in coronary artery.
- Investigation of the effect of LEVO in relation to the simulated transmural pressure of the vessel wall.

2. MATERIALS AND METHODS

2.1 MEASUREMENTS OF ISOMETRIC TENSION IN CONDUIT AND CAPACITANCE TYPES OF BLOOD VESSELS

2.1.1 Investigations of BK_{Ca} channel

2.1.1.1. Canine coronary arterial preparations

Mongrel dogs of either sex, weighing, 9-15 kg were anesthetized with sodium pentobarbitone (30 mg/kg, i.v.) and heparinized (1000 IU/kg). The heart was excised and placed into a Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 120, $NaHCO_3$ 20, KCl 4.1, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and glucose 11 (pH 7.4). Rings (1.1-1.9 mm o.d., 5 mm width) from the descending and circumflex branches of the left coronary artery were isolated from the heart. Endothelium was removed by gently rubbing the endothelial surface with a stainless steel wire covered with a cotton swab. Preparations were then mounted in water-jacketed baths containing 2 ml KHS bubbled with 95% O_2-5% CO_2 gas mixture at 37 °C, and that will be used also in the following investigations. The isometric tension was recorded with a force-displacement transducer (Hugo Sachs Elektronic, Type F30, Germany, Fig. 1) and mechanical responses of arterial rings were displayed by means of a pen recorder (Type 175, KUTESZ, Hungary) as in the following investigations in conductance and capacitance vessels. Rings were stretched up to 10 mN and allowed to stabilize for 45 min. This tension was readjusted to 10 mN during equilibration.

Following equilibration, contractions were induced by 25 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and at the maximum amplitude of contraction 1 μM acetylcholine (Ach) was applied. Only those arterial preparations were used for the experiments that responded with contraction after addition of 1 μM Ach. This protocol served as evidence for functionally de-endothelialized arterial preparations. indomethacin (INDO, 10 μM) was used in all experiments to exclude the effect of endogenous prostaglandins on the arterial tone.

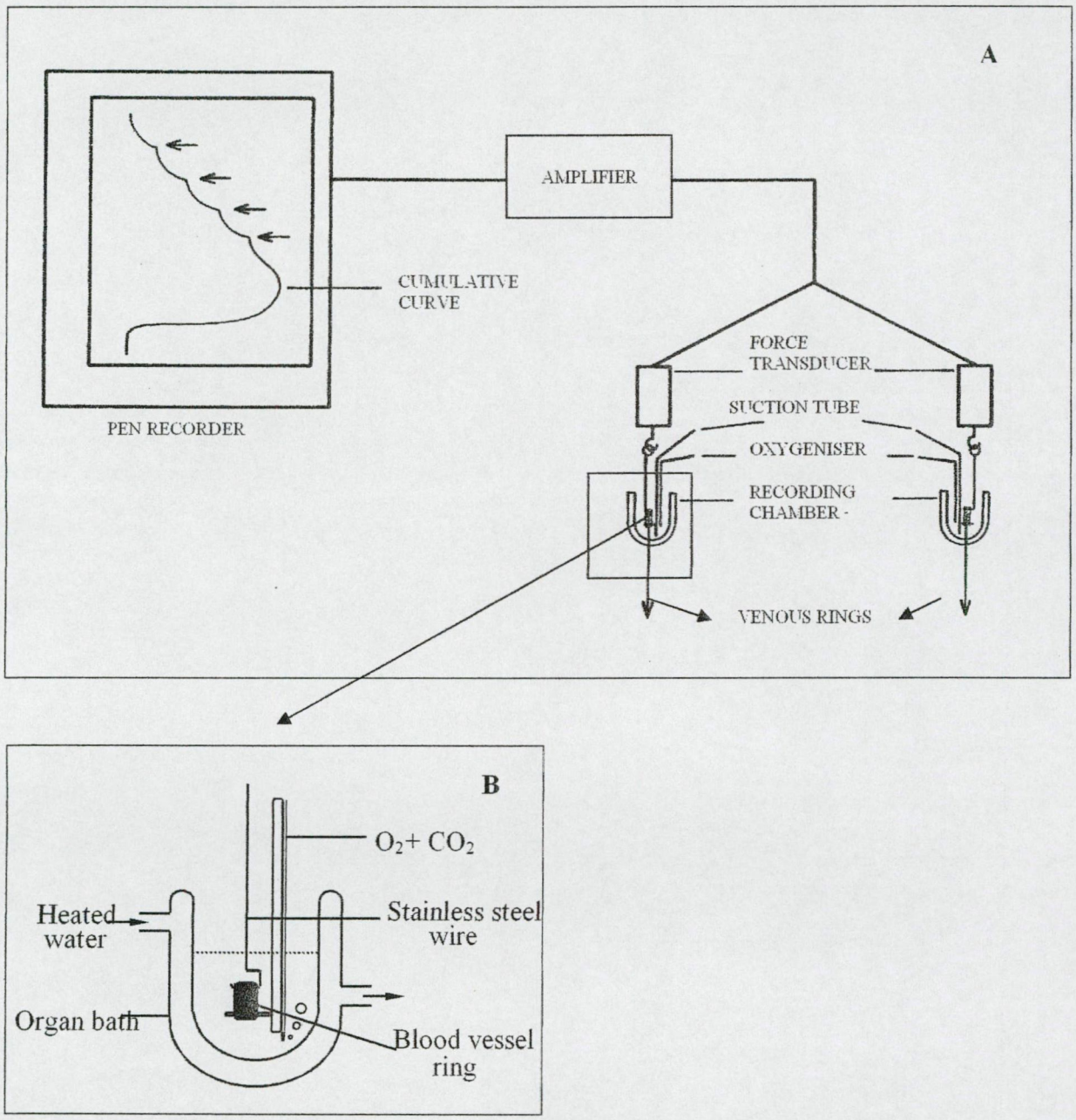

Figure 1. Schematic figure of measurement of isometric tension (A) and organ bath (B)

Two parallel rings isolated from the same branch of a coronary artery were used for measurement of contraction and relaxation. After checking the functional denudation with Ach, the rings were washed with KHS. One arterial ring was exposed to solvent and served as a control while the other was exposed to a potassium channel blocker [90 nM iberiotoxin (IBTX) or 30 μM glibenclamide (GLI)] for 30 min. Contractions were induced again by the addition of 25 μM $PGF_{2\alpha}$ to both rings. At the steady state of contraction cromakalim (CRO) was applied in cumulative manner. The same arterial rings were used for further contractions.

In another series of experiments the contraction of the rings was induced with low (14.4-20.4 mM) or higher (35.4-40.4 mM) concentrations of depolarizing potassium chloride (KCl). Elevation of potassium concentration in the solution was made by substituting NaCl with equimolar KCl in the KHS medium. Experiments were started with 14.4 mM KCl medium and, if it was necessary, replaced with higher concentrations of KCl until it was enough to induce contraction. Relaxation by exogenous nitric oxide (NO) in the presence and absence of 90 nM IBTX was examined.

Preparation of NO solution (about 1.6 mM): a saturated solution in double-distilled water was prepared using a slight modification of a previously described method (Menon et al. 1991). Water in a 10-ml vacutainer tube was deoxygenated by purging with 100% nitrogen for 1 hour and then bubbled with nitric oxide for 20 min. For diluting nitric oxide, 100 µl of this solution was transferred with a gas tight syringe (Hamilton, Bonaduz, Switzerland) to another tube containing 10 ml deoxygenated water and used for experiments within 1.5 h. Relaxation with nitric oxide (100-800 nM) was induced at the steady state of KCl contraction in control rings and in rings pretreated with IBTX as described above.

2.1.1.2. Human saphenous vein preparations

Vena saphena magna was obtained from patients suffering from varicose vein disease and undergoing surgical intervention. The experimental protocol complied with the Declaration of World Medical Association proclaimed in Helsinki and was approved by the Human Ethical Review Board of the Albert Szent-Györgyi Medical University (No. 164/2002 OEj). Visually intact parts of the saphenous veins were carefully excised and placed into an ice-cold KHS solution. Rings of 5 mm width were cut from the vein. After cleaning from the connective tissue rings were mounted in parallel in two water-jacketed baths, data handling occurred as described above (2.1.1.1.). The rings were stretched up to 8-10 milliNewton (mN) and allowed to stabilize for 30 min. This tension was continuously readjusted to the basal tension during equilibration.

NO was obtained by reducing $NaNO_2$ in the following medium: (140 mM Na_2SO_4, 100 mM NaI and 270 µl concentrated H_2SO_4 in 50 ml double distilled water. This solution was bubbled with 95% N_2 and 5% CO_2 gas mixture for 45 min in an air-tight vacutainer tube. $NaNO_2$ was injected into this O_2 free solution to result in 100 µM stock solution.

After 30 min equilibration of the two venous preparations with 90 nM IBTX and with the corresponding volumes of solvent contractions were induced with 5-hydroxytryptamine

(5-HT, 0.125 nM). At the steady-state contractions cumulative concentrations of NO (50-1550 nM) were applied.

2.1.2 Investigations for K_V channel

2.1.2.1. Human coronary arterial preparations

Human coronary arteries were prepared from parts of undiseased donor hearts unsuitable for transplantation from which the aortic and pulmonary valves had been previously excised for homograft-valve surgery. Before implantation of the hearts the patients did not receive any medication except for dobutamine, furosemide and plasma expanders. The experimental protocol complied with the Declaration of World Medical Association proclaimed in Helsinki and was approved by the Human Ethical Review Board of the Albert Szent-Györgyi Medical University (No. 51-57/1997 OEj). The hearts were stored in a cardioplegic solution, which had the following composition (in mM): NaCl 110, KCl 16, $MgCl_2$ 16, $CaCl_2$ 1.2, $NaHCO_3$ 10, at 4 °C and used for experiments within 12 hours. The carefully removed human epicardial coronary arteries were cleaned from the connective tissue and the blood vessels were cut into 5 mm rings. Then rings were placed into the organ bath, data handling took place as describerd above (2.1.1.1.). Rings were stretched up to 10 mN, and equilibrated for 45 min (the medium was changed in every 15 min).

Concentration-response curves to 4-aminopyridine (4-AP, 12.5 nM-187.5 μM) were obtained using the drug in a cumulative manner in the organ bath after the equilibration time. After the 4-AP contraction had been completed 0.1 and 1 μM nisoldipine (NIS) were added.

2.1.2.2. Canine saphenous vein preparations

The experiments (and also further investigations on animals) were performed by the permission of the Local Committee for Animal Research of Albert Szent-Györgyi Medical University (No. 71/1999). Mongrel dogs of either sex were heparinized (500 IU/kg iv) and anesthetized with sodium pentobarbital (30 mg/kg iv). Lateral saphenous veins were taken out, carefully cleaned from connective tissue and cut into 5 mm ring segments for investigation. Experiments were performed in the absence of vascular endothelium. Removal of the endothelium was achieved mechanically by using a cotton swab on a glass rod. Functional endothelial denudation was evidenced by lacking vascular relaxation after administration of 1 μM bradykinin (BRK). Paired venous rings were investigated in parallel. Vein segments were mounted in isolated organ baths and data handling occurred as described above (2.1.1.1.).

The 5-mm ring segments were stretched up to 10 mN and equilibrated in 2 ml KHS for 45 min. After the equilibration of the venous rings under 10 mN stretch concentration-response curves to 4-AP (0.0125-5 μM) were obtained by adding the drug to the tissue bath in a cumulative manner. Following the maximum contraction induced by 4-AP the preparations were washed three times and incubated with 1 mM noradrenaline (NA) for 10 min. Loading procedure of the preparations with NA involved the addition of 1 mM NA for 10 min followed by thorough washing out (at least three times) of the catecholamine from the organ bath. After washing out this large concentration of NA, the changes of tone were monitored again by repeated exposure to 4-AP.

In parallel experiments 6-hydroxydopamine (6-OHDA, 0.5 mM, 3 h incubation) was used to destroy the noradrenergic nerve endings before the addition of 4-AP.

2.1.3. Investigations with levosimendan

2.1.3.1. Porcine coronary arterial preparations

Porcine hearts were obtained from the local slaughterhouse and transported to the laboratory in ice-cold KHS. The experimental protocol was approved by the Ethical Review Board of the University of Szeged, Hungary (I-74-3/2002.MÁB.sz.). Epicardial coronary arteries of the descending branch were used for the experiments. 5 mm-long ring segments were cut out of the coronary artery, cleaned from the surrounding connective tissue and the endothelium was mechanically removed (denudation procedure in the case of canine saphenous vein (2.1.2.2)). Then two rings were submerged into water-jacketed baths for isometric tension recordings. Only those coronary preparations were used for the experiments that responded with contraction to the addition of 1 μM BRK. Two coronary rings of each porcine heart were mounted separately in isolated tissue baths and data handling took place as described above (2.1.1.1.) The preparations were stretched up to 30 mN (because the wet weight of the porcine coronary preparation is three fold heavier than that of the human ones) and allowed to equilibrate for 90 min. Within this equilibration period the incubation medium was changed in every 15 min and a response to 30 mM potassium chloride (KCl) rich solution was produced in order to stabilize the contractile activity of the blood vessels. In experiments designed to determine if CRO and LEVO caused the porcine isolated coronary artery to relax, after equilibration of the pairs of ring segments, contractions to 20 mM KCl were induced. An additional 15.8 mM KCl was added to the medium because the KHS had already contained

4.2 mM KCl. When the steady-state contraction amplitude produced by KCl had developed, one of the rings was cumulatively exposed to LEVO (0.009-3.2 μM) or CRO (0.0125-5 μM). The other ring was at first preincubated with 1 μM GLI 30 min before the addition of the contractile agent, KCl, and the protocol was then followed by the cumulative administration of either LEVO or CRO. 2 μl solvent of GLI was without effect on the parallel ring.

In another series of experiments the capacity of the other potassium channel blocker, tetraethylammonium (TEA), to influence relaxations caused by CRO and LEVO was assessed. The protocol was similar to that described above, except that the concentration-response curves for both LEVO and CRO were determined in the presence of 2 mM TEA and of the corresponding solvent (20 μl distilled water) of the potassium channel blocker (5 min preincubation before addition of KCl).

In a third series of experiments the ability of the K_{Ca} channel blocker, 100 nM IBTX (30 min before KCl), and that of the K_v channel inhibitor, 0.5 mM and 5 mM 4-AP (10 min before KCl) to influence the coronary relaxations by LEVO was investigated. During this protocol, a parallel control with the solvents of the potassium channel blockers (20 μl distilled water of IBTX and 20-200 μl of 4-AP) was also investigated.

2.1.3.2. Human internal mammary artery preparations

Internal mammary arteries (IMA) were obtained atraumatically from patients who were operated on due to coronary occlusion, healed with mammarian graft insertion. The experimental protocol complied with the Declaration of World Medical Association proclaimed in Helsinki and was approved by the Human Ethical Review Board of the Albert Szent-Györgyi Medical University (No. 51-57/1997 OEj). Before explantation of the vessels patients had got usually medication as follows: β-adrenergic blocking agents, angiotensin converting enzyme inhibitors, Ca antagonists and nitrates.

The carefully removed internal mammary arteries were cleaned from the connective tissue, and the endothelium was removed by carefully rubbing the endothelial surface with a stainless steel wire covered with a cotton swab. Then vessels were cut into 5 mm rings and were placed into the organ bath and data handling occurred as described above (2.1.1.1.). Rings were stretched up to 23 mmHg and 46 mmHg, according to the Laplace law [$(P=T_W*2\Pi/I$, where P is the intraluminal pressure, T_W is wall tension calculated from the initial stretch applied (F) and from length of vessel segment (L), that is T_W= F/L (mN/mm2), and I is internal circumference of the vessel wall (mm), values obtained in Pascal were

converted into mmHg)], and equilibrated for 45 min. After equilibration time rings were contracted with NA (1-7 μM) cumulatively, and at the end point of the maximal tone LEVO was cumulatively added into the organ bath (0.3-0.6 μM). After all, the organ bath was washed out three times with KHS, and the experiment was repeated at 92 mmHg prestretched vessels. After repeated washing out, the functionally damaged endothelium was proved to have no effect of 0.1 μM BRK on the tone.

2.1.3.3. Canine saphenous vein preparations

Lateral canine saphenous veins were used in this protocol. The method was similar to that written in chapter 2.1.2.2. In the first series of experiments, two parallel rings were mounted in separated water jacketed baths; one of them was stretched up to 5mN, the other one to 10mN. These values of intraluminal stretching were considered as moderate and high venous blood pressures (10 and 20 mmHg) according to the calculations by the Laplace law (2.1.3.2.).

After 45 min equilibration, at the steady state contraction caused by 3.25μM NA, LEVO was cumulatively administered into the organ bath (0.03-3.4 μM).

In the second series of experiments the two parallel measurements were performed at the same level of vascular tone. At the first third of the equilibration 1.5μM GLI was added to one of the rings and corresponding solvent to the other one. After another 30 min NA contraction was also induced.

2.1.3.4. Human portal vein preparations

Portal veins were prepared from liver transplants of patients, aged 17 to 62 years (average age 44± 14 years) who had died accidentally. Segments (2.2 cm long) of portal vein were removed from those livers deemed unsuitable for transplantation and were stored for up to 4 h in Bretschneider solution: (NaCl (15 mM), KCl (9mM), potassium hydrogen-2-oxoglutarate (1 mM), $MgCl_2x6H_2O$ (4 mM), histidine HClx H_2O (18 mM), histidine (180 mM), tryptophan (2 mM) and mannit (30 mM). Only macroscopically healthy veins were used. Veins were placed into an ice-cold KHS. The venous segments were prepared in the clinical surgery and transported to the experimental laboratory within 15 min. The vessels were then dissected free of perivascular tissue and cut into rings (5 mm long).

Two rings of each portal vein were mounted separately in water-jacketed baths and data handling occurred as described above (2.1.1.1.). Preparations were stretched until near-maximum contractile responsiveness to NA was reached at 40 mN basal tension. Each ring was allowed to equilibrate for 45 min before initiation of experimental procedures and during this period the incubation medium was changed every 15 min and the resting tension was readjusted.

In experiments designed to determine if CRO and LEVO cause the human isolated portal vein to relax, after equilibration of the pairs of ring segments, contractions to 10 μM NA were induced. When steady-state contraction amplitude produced by NA had developed, one of the rings was cumulatively exposed to LEVO (0.01-1.27 μM) and the other ring was treated with the corresponding volumes of the solvent (1-64 μl). The same procedure was used with another pair of venous rings obtained from the same liver, except that, instead of LEVO, CRO (0.2-47 μM) and its solvent (0.5-125 μl) were administered cumulatively at the steady-state of NA-induced contraction. In the latter case, the largest volume of solvent produced relaxations in some cases an effect which was deduced from comparisons of the magnitude of relaxation induced by CRO.

In another series of experiments the capacity of GLI (1.5 and 15 μM) to decrease relaxations caused by CRO and LEVO was assessed. The protocol was similar to that described above, except that concentration-response curves for either CRO or LEVO were determined in the presence and absence of 1.5 μM GLI. One of the portal rings was incubated with the low concentration of GLI, the other ring with the solvent of the blocker 30 min before the addition of NA. After completing the agonist-response curves for CRO and LEVO, the ring not treated with GLI was washed three times and incubated with 15 μM GLI for 30 min. Then steady-state contraction was induced by NA and another agonist-response curve was obtained.

2.1.3.5. Human saphenous vein preparations

Vena saphena magna was obtained from patients suffering from varicose vein diseases, as it was described before (2.1.1.2.), but the equilibration time was 45 min.

After 45 min equilibration, 15 μM GLI was added to one of the rings and 30 μl solvent of GLI to the other one. After 30 min incubation, contraction was induced by exposing the two parallel venous preparations to 0.125 μM 5-HT. At the steady-state contraction, 0.04-2.8 μM concentrations of LEVO were cumulatively applied. LEVO was usually added to the tissue bath at intervals of 5-6 min.

In another series of experiments, instead of GLI, 90 nM IBTX or its solvent (18 µl double distilled water) were used for preincubation of the venous rings for 30 min. Then, LEVO, in concentrations of 0.04-2.8 µM, was cumulatively added to the preparations.

2.2. DATA ANALYSIS AND STATISTICAL COMPARISONS

The decrease of the venous tone caused by NO, CRO or LEVO was expressed as the percent of the 5-HT, KCl, $PGF_{2\alpha}$, or NA-induced steady-state contraction amplitudes. Results are expressed as mean ± s.e.m. and n refers to the number of venous or arterial rings obtained from different animals or human organs. One-way analysis of variance with repeated measures was used to determine if significant differences existed between groups. When analysis of variance showed significant differences, the Newman-Keuls test was performed to determine differences between individual values. Values for 50 % effective concentration (EC_{50}) were obtained by fitting the exponential equation of $100/\{1+\exp [b*(x-c)]\}$ to individual values, in that 'b' is the slope and 'c' is EC_{50} of the concentration response curve. In another experiments $a*x/(x+b)$ was fitted to concentration response curves, where 'a' is maximum (E_{max}) and 'b' is the EC_{50} value of the curve.

2.3. DRUGS AND OTHER AGENTS

Drugs used in the present study were the following:

KHS solution (composition in mM: NaCl 120, $NaHCO_3$ 20, KCl 4.1, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and glucose 11 (pH 7.4) Reanal, Budapest, Hungary), nitric oxide, $NaNO_2$, Na_2SO_4, NaI, H_2SO_4, 4-aminopyridine, 5-hydroxytryptamine, 6-hydroxydopamine hydrobromide, acetylcholine, creatinine sulfate complex, glibenclamide, , indomethacin, L-noradrenalin-L-bitartrate, prostaglandin $F_{2\alpha}$ (Sigma, St. Louis, MO), NG-nitro-L-arginine (L-NOARG), tetraethylammonium HCl, sodium nitrite, pentobarbital sodium (Serva). Cromakalim was obtained from Beecham Pharmaceuticals (Harlow, UK). Iberiotoxin was synthesized by Gábor K. Tóth (Dept of Med Chemistry, Szeged, Hungary), and it was dissolved in double-distilled water to give a concentration of 3 µM.

Levosimendan was a gift from Orion-Farmos, Espoo, Finland., and it was prepared daily by dissolving it in 70% ethanol (0.21mM in stock solution). CRO and GLI were dissolved in double distilled water containing 20% dimethylsulfoxide and 20% ethanol. The concentrations of CRO and GLI were 0.45 mM and 1 mM in the stock solutions, respectively.

TEA, IBTX and 4-AP were dissolved in distilled water and the concentrations of stock solutions were: 1 M, 10 μM and 50 mM, respectively.

NA was dissolved in 0.9% NaCl with 10 mM ascorbic acid, resulting in 1 mM NA in the stock solution. PG $F_{2\alpha}$ was dissolved in 70% ethanol (stock solution 10.5 mM). Indomethacin was dissolved in 96% ethanol at a concentration of 1 mM.

3. RESULTS

3.1. Investigations of the effect of potassium channel blockers on BK_{Ca} channel

3.1.1. Effect of CRO and NO on canine coronary arteries

3.1.1.1. Effect of CRO in the absence and presence of GLI. Functional BK_{Ca} channel differs from the K_{IR} (K_{ATP}) channel.

The activator of K_{ATP} channels, CRO, produced a dose-dependent relaxation of coronary arterial rings in a concentration range of 0.15-9.6 μM (Fig. 2.). The calculated EC_{50} of CRO was 0.41 μM in the absence of GLI. Preincubation of coronary rings for 30 min with 30 μM GLI did not affect the resting tone of the arteries (control=0.8±1.1%, GLI=1.7±2.0%, n=7, p>0.05; percent increase of tone compared with the amplitude of steady-state contraction induced by $PGF_{2\alpha}$) nor was there an effect on the magnitude of contraction induced by $PGF_{2\alpha}$ (control=49.4±6.0 mN, GLI=53.8±7.4 mN, n=7, p>0.05). However, GLI almost completely inhibited the relaxation induced by CRO.

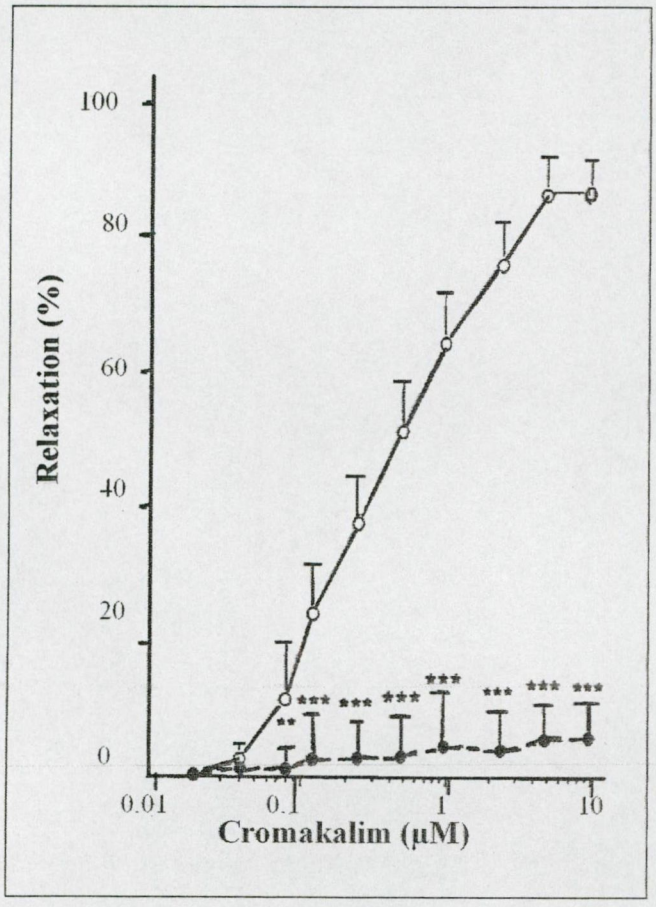

Figure 2. Effect of GLI on the relaxant responses to CRO in isolated rings of canine coronary arteries. Paired arterial rings prepared from the same heart were pretreated with either 30μM GLI (•) or the corresponding volume of vehicle (○) 30 min before addition of 25μM $PGF_{2\alpha}$. At the steady-state contraction induced by the $PGF_{2\alpha}$, CRO was applied cumulatively. Each value represents the mean of percent relaxation obtained in seven coronary rings from different dogs. Vertical lines show the s.e.m. $^{**}P<0.01$, $^{***}P<0.001$ compared with vehicle-treated group.

3.1.1.2. Effect of CRO in the absence and presence of IBTX.

After 30 min preincubation with 90 nM IBTX, the specific inhibitor of BK_{Ca} channels, the resting tone of the arteries was slightly but significantly increased compared with control (control=1.2±1.2%, IBTX-treated= 10.4±2.5%, n=7, $p<0.05$). The maximum contraction amplitude produced by $PGF_{2\alpha}$ did not differ between the IBTX-treated group and the control (control=55.6±5.3 mN, IBTX-treated=53.3±7.8 mN, n=7, $p>0.05$). Preincubation of coronary arteries with IBTX for 30 min did not change the vasodilating potency of CRO (EC_{50} CRO=0.43 μM, EC_{50} CRO+IBTX=0.42 μM, Fig. 3.).

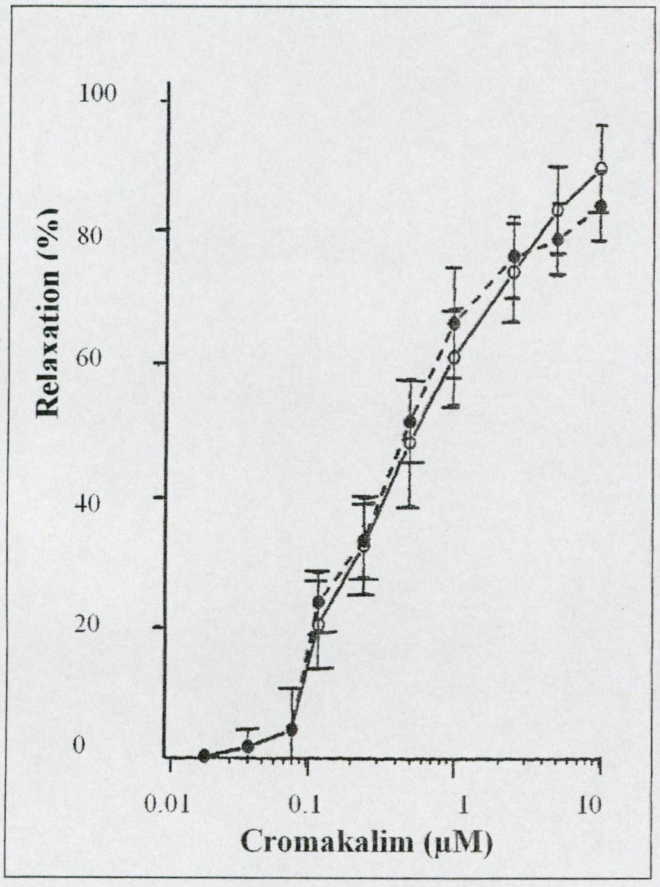

Figure 3. Effect of IBTX on the relaxation induced by CRO in isolated rings of canine coronary arteries. One of two arterial rings was pretreated with 90 nM IBTX (•) and the other was exposed to the solvent of IBTX (○). After 30 min preincubation with IBTX, contraction was induced with 25 μM $PGF_{2\alpha}$. At the steady-state of contraction, CRO was added in a cumulative fashion. Values are mean±s.e.m. each representing seven coronary preparations obtained from different dogs.

3.1.1.3. Effect of exogenous NO on KCl depolarized coronary rings.

Threshold concentration of KCl necessary to induce contraction varied between 14.4 and 20.4 mM. When the KCl concentration was 14.4-20.4 mM, IBTX significantly enhanced the amplitude of contraction (control=11.6±4.3 mM, KCl=25.3±3.7mM, n=7, $p<0.05$). The results obtained with NO are summarized in Table 1. Relaxation by 100 and 200 nM NO was significantly inhibited by IBTX. The effect of higher concentrations (400-800 nM) of NO was not influenced by the toxin. Depolarization of the artery with 35.4-40.4 mM KCl resulted in a

contraction amplitude of 35.7±7.0 mN (control) that did not differ from that of the IBTX pretreated group (38.4±4.7 mN, n=6, $P>0.05$ compared with control). Amplitudes of relaxation by NO were significantly smaller at high KCl concentrations compared to low ones. The inhibitory effect of IBTX on NO-induced relaxation disappeared at 35.4-40.4 mM of depolarizing KCl.

Effect of IBTX on NO-induced relaxation in depolarizing KCl solution.

Agonist		Relaxation (%)		
Nitric oxide (nM)	100	200	400	800
14.4-20.4 mM KCl	30.0±5.2	50.8±4.6	71.3±3.6	89.6±4.1
+IBTX	12.0±4.9**	28.3±6.0**	67.8±5.5	92.5±3.3
35.4-40.4 mM KCl	3.2±2.0^{++}	13.4±4.1^{++}	41.3±6.90^{+}	67.4±6.6^{+}
+IBTX	5.3±3.3	17.2±5.2	46.0±4.7	59.1±5.7

Data are mean ±s.e.m. Number of experiments was six in the low-potassium medium and seven in the high-potassium medium. **$P<0.01$ compared with the corresponding 14.4-20.4 mM KCl. $^{+}P<0.05$, $^{++}P<0.01$ compared with values obtained in 14.4-20.4 mM KCl.

Table 1. Effect of IBTX on NO-induced relaxation in depolarizing KCl solution.

3.1.2. Effect of NO on the IBTX-sensitive potassium channel in human saphenous vein

NO in a concentration range of 50-1550 nM dose-dependently relaxed both IBTX pretreated and control saphenous vein rings. Significant differences have been found between the corresponding values of NO and IBTX+NO (Table 2). In the IBTX pretreated group the calculated EC_{50} values of NO was 1471.9 nM and it was 952.7 nM in the control group, respectively.

Relaxation (%)					
Nitric oxide (nM)	50	150	350	750	1550
5-HT	1.01.0	5.5±1.2	16.8±4.2	29.9±3.1	65.5±6.9
5-HT+ IBTX	0.0±0.0	0.0±0.0*	4.5±1.7*	15.4±3.6*	34.8±7.3*

Table 2. Effect of IBTX on NO-induced relaxation of human saphenous vein. Number of preparations=6, Data are mean ± s.e.m., *P<0.05 between the corresponding values of 5-HT and 5-HT+IBTX.

3.2. Investigations of the effect of potassium channel blockers on K_V channel

3.2.1. *Effect of 4-AP on human isolated coronary arteries*

The K_V inhibitor, 4-AP enhances the basal tone and could induce contraction in the human coronary artery in a concentration dependent manner. The contraction amplitude was inhibited by a voltage dependent calcium channel inhibitor, NIS (Fig. 4.). NIS was able to reverse, at least in part, the contractile effect of 4-AP. This result proves the potassium channel blocking property of 4-AP. Potassium channel block leads to depolarization with consequent increase in open probability of voltage dependent calcium channels.

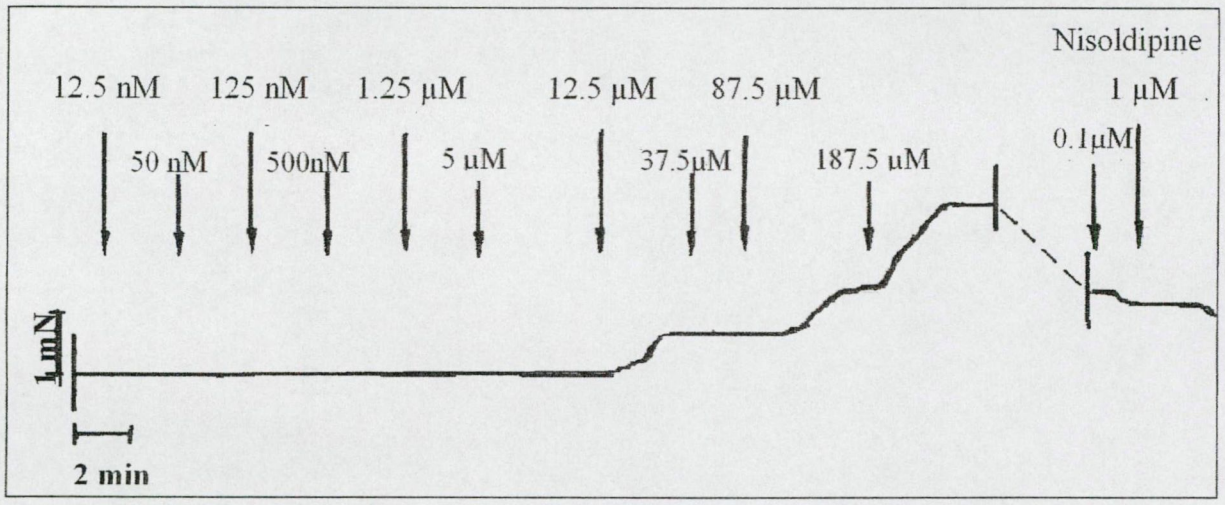

Figure 4. Representative figure, effect of NIS on 4-AP contracted human coronary artery

3.2.2. *Effect of 4-AP on the basal tone of canine saphenous veins*

4-AP, in concentrations of 0.0125-5 μM, caused contractions in canine isolated saphenous vein preparations in the absence of endothelium In the absence of NA, the maximum contractile response was small (1.3±0.57 mN, n=6) and the EC_{50} value for 4-AP was 2.02±0.53 μM. After loading the preparations with 1 mM NA, accumulation of NA in noradrenergic nerves of the venous tissues significantly increased the contractions in response to repeated administration of 4-AP (Fig. 5.). Under these conditions, the maximum contraction of the venous rings increased about eight-fold (10.51±3.64 mN, n=9, $p<0.05$) compared to the non-loaded tissues. The EC_{50} value was even smaller than in the venous preparations without NA filling (0.61±0.3 μM, $p<0.05$). To detect the role of perivascular nerves in the uptake of NA, we destroyed the neuronal vesicles with 6-OHDA (Fig. 5.). After this chemical denervation, 4-AP failed to cause contraction up to the largest concentration applied (5μM) in canine saphenous vein.

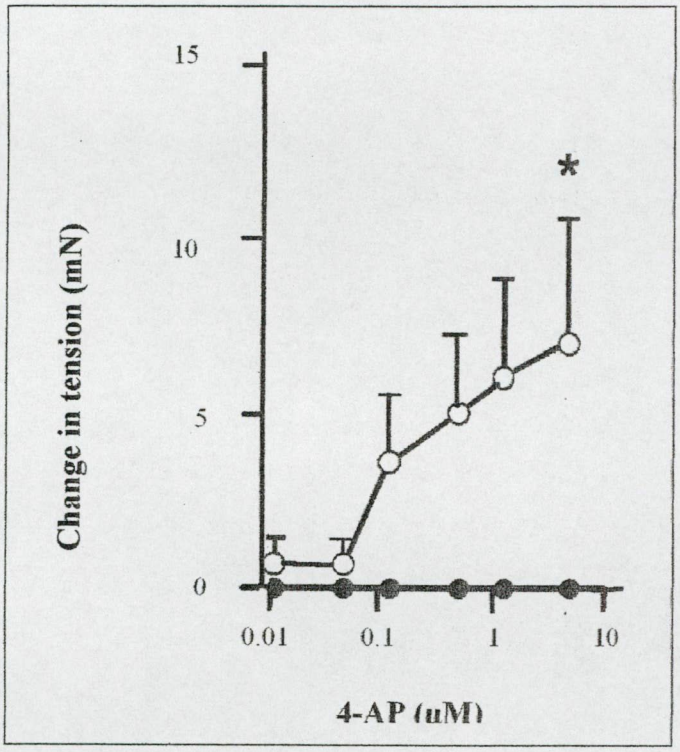

Figure 5. Effect of 6-OHDA on the contractions induced by 4-AP in canine saphenous vein in the absence of endothelium. Open circles represent the effect of 4-AP. Closed circles denote the effect of 4-AP after incubation the venous rings with 0.5 mM 6-OHDA. Number of experiments was seven with and without 6-OHDA. *$P<0.05$ between corresponding values of 4-AP and 4-AP+6-OHDA. Mean values are given together with s.e.m.

3.3. Investigations of the effect of levosimendan

3.3.1. Effect of levosimendan in porcine coronary arteries

3.3.1.1. Effect of GLI on the coronary dilating action of CRO and levosimendan

CRO and LEVO caused a concentration dependent relaxation on the KCl precontracted coronary artery preparations (Fig. 6A and B). 1 μM CRO almost abolished the 20 mM KCl-induced contraction (93.9±6.9% of the KCl-induced tone, Fig. 6A), an effect which was not significantly enhanced further by the increase of the concentration of the drug to 5 μM (Emax: 101.5±7.1%). 3.2 μm LEVO completely relaxed the KCl-evoked contraction (Fig. 6B). On the basis of the EC_{50} values, CRO was 4.1-fold more potent (0.10±0.02 μM, n=5) than LEVO (0.41±0.10 μM, $P<0.01$ compared to CRO, n=7) under identical experimental conditions in the absence of GLI. 1 μM GLI did not significantly affect the contractions induced by KCl (control: 57.7±7.1 mN and GLI: 63.2±6.9 mN, n=12). This K_{ATP} blocking drug decreased the maximum relaxation of CRO (Emax: 47.3±10.7% at 5 μM, Fig. 6A) while it did not influence the LEVO-induced decrease of coronary tone up to 3.2 μM of the inodilator (Emax without GLI: 97.8±8.4%; Emax with GLI: 98.6±10.1%; Fig. 6B). The presence of GLI resulted in a 4.6-fold decrease of the potency value for CRO (EC_{50}: 0.46±0.02 μM, $p<0.01$ compared to CRO alone, n=5) whereas it did not change significantly the potency value calculated for LEVO (EC_{50} 0.43±0.07 μM, compared to LEVO alone, n=7).

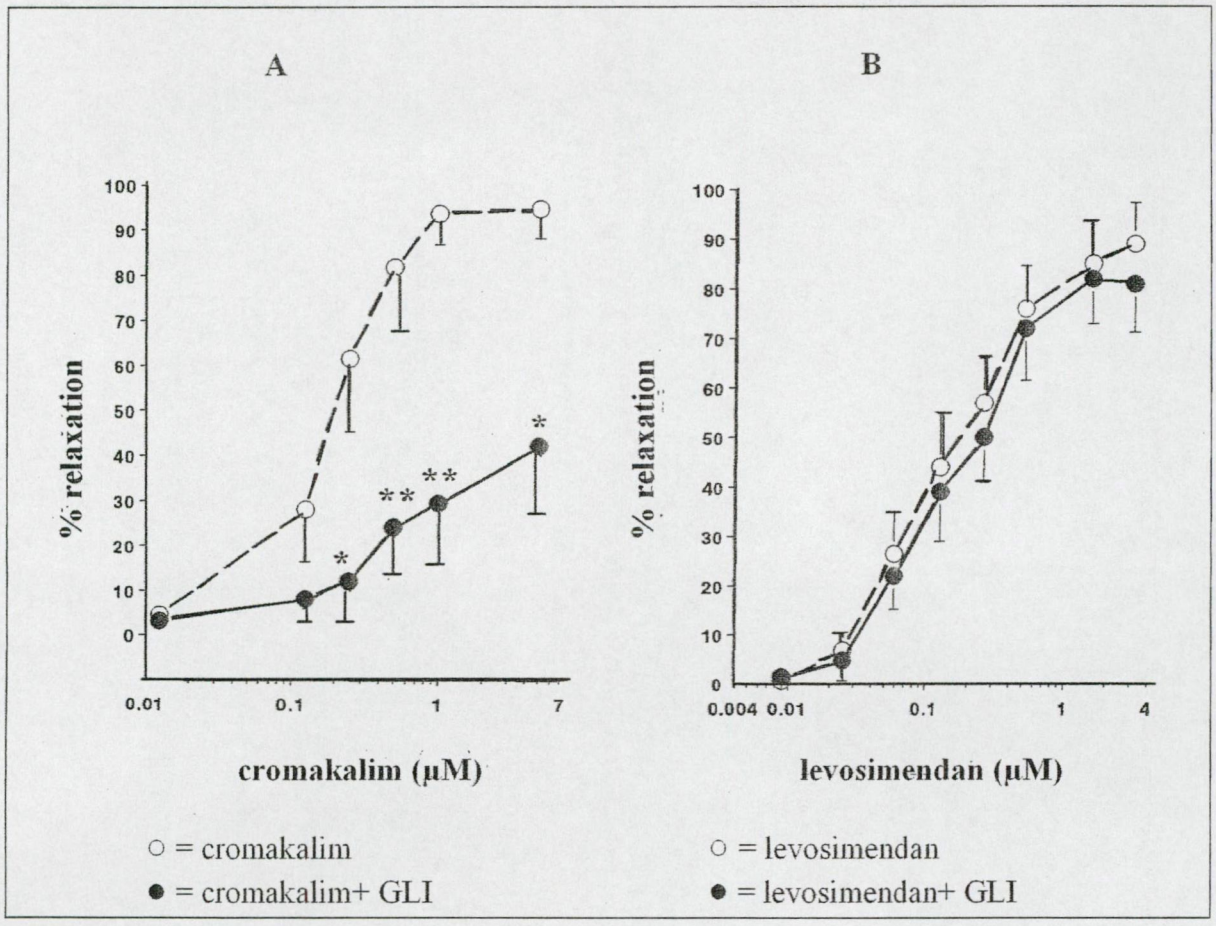

Figure 6. Effect of CRO (A) and LEVO (B) on the tone of porcine isolated left descendent coronary artery in the absence (○) and presence (●) of 1 μM GLI. Experiments were performed in 20 mM potassium chloride-precontracted coronary rings without functional endothelium. Magnitude of relaxations was expressed as percent of KCl-induced tone. Data represent mean±s.e.m. of 5 (A) and 7 (B) independent experiments. *P<0.05, **P<0.01 compared with the corresponding values of GLI treated coronary preparations.

3.3.1.2. Effect of TEA on the coronary dilating action of CRO and levosimendan

The next series of experiments was aimed at determining the capacity of TEA to modify the relaxations induced by CRO and LEVO (Fig. 7A and B). 2 mM TEA pretreatment for 5 min did not influence significantly the contractile amplitude of the coronary preparations induced by 20mM KCl (control: 64.4±7.8 mN, TEA: 73.1±7.6 mN, n=6) and caused no change in the CRO concentration-response curve (Fig. 7A). Neither the magnitudes of the relaxations nor the potency values showed considerable differences in the absence and presence of TEA (Emax without TEA: 97.7±9.3%, EC_{50}: 0.16±0.02 μM, n=6; and Emax with TEA: 103.6±8.8%, EC_{50}: 0.18±0.03 μM, n=6).

The K_{Ca} channel blocker did not change the contractile amplitude induced by 20 mM KCl when the effect of LEVO was studied (control: 67.9±5.9 mN, TEA: 64.9±6.2 mN, n=8). However, TEA decreased the calculated maximal relaxation of LEVO (Emax without TEA: 96.6±7.8%, Emax with TEA: 73.8±8.1%, $p<0.05$, n=8, Fig. 7B). This inhibitory effect was evident only at large concentrations of LEVO, i.e. at 1.6 and 3.2 μM of the inodilator drug. The EC_{50} of LEVO did not differ significantly in the absence and presence of TEA (0.24±0.05 μM and 0.16±0.04 μM, respectively, n=8).

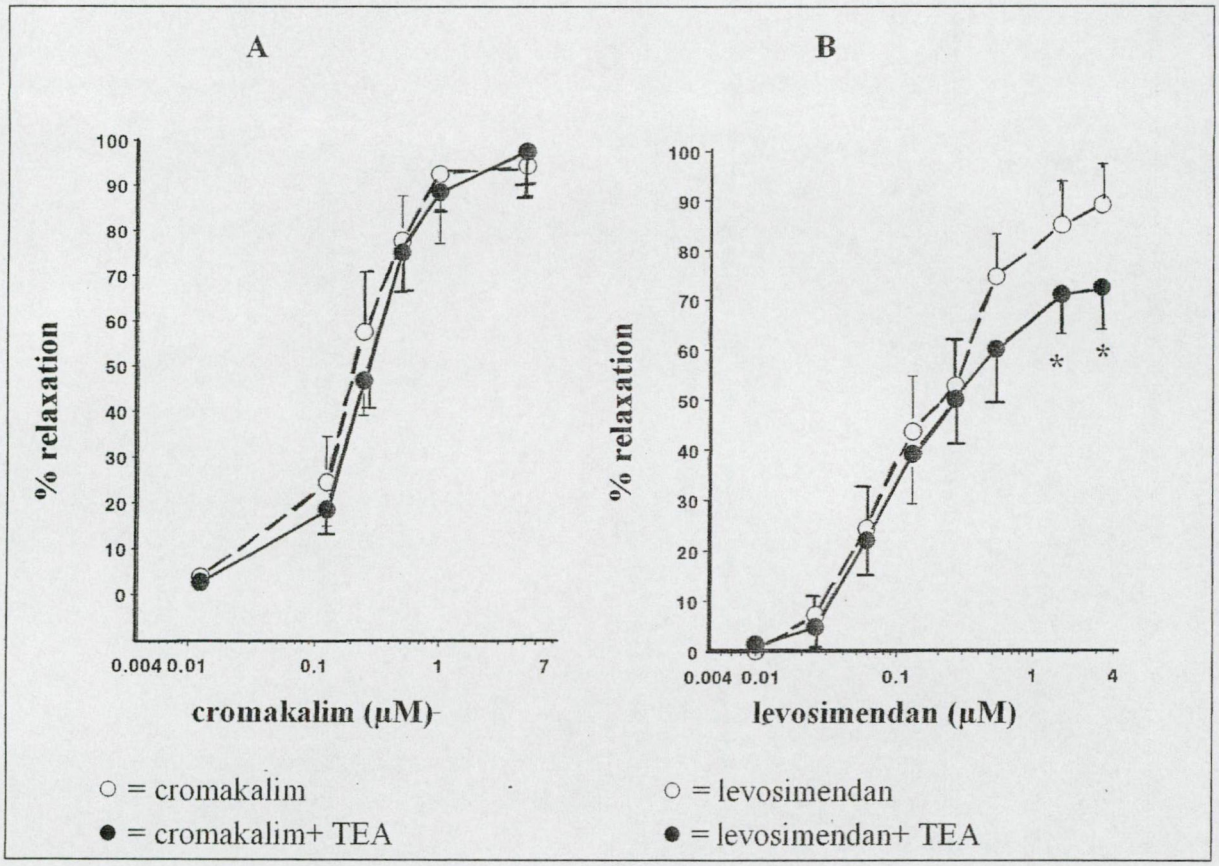

Figure 7. Effect of CRO (A) and LEVO (B) on the porcine isolated left descendent coronary artery in the absence (○) and presence (●) of 2 mM TEA. Experiments were performed in 20 mM KCl- precontracted coronary rings without functional endothelium. Magnitude of relaxations was expressed as percent of KCl-induced tone. Data represent mean±s.e.m. of 6 (A) and 8 (B) independent experiments. *$P<$ 0.05, compared with the corresponding values of TEA treated coronary preparations.

3.3.1.3. Effect of IBTX and 4-AP on the coronary artery dilation induced by levosimendan

In third series of experiments another K_{Ca} channel blocker, IBTX, and the K_V channel blocker, 4-AP, were investigated on the coronary artery relaxing effect evoked by LEVO (Fig. 8). 100 nM IBTX pretreatment of the coronary preparations for 30 min did not change the contractile effect of 20 mM KCl (control: 75.7±9.9 mN, IBTX: 73.7±6.6 mN, n=8). This K_{Ca} channel blocker caused a small but significant decrease of the maximal effect of the inodilator (Emax without IBTX: 91.7±3.1%, Emax with IBTX: 76.8±4.4%, $P<0.01$, n=8, Fig. 8A). However, the potency of the inodilator was not changed by IBTX (LEVO EC_{50}: 0.48±0.07 μM, LEVO+IBTX EC_{50}: 0.46±0.04 μM). 10 min incubation of the coronary rings with the K_V channel inhibitor, 4-AP (0.5 mM), did not significantly change the contractile sensitivity to KCl (control: 56.9±9.0 mN, 4-AP: 49.8±7.6 mN, n=7), but it decreased the coronary artery relaxation induced by LEVO which was significant at as low as 0.5 μM concentration of the inodilator drug (Fig. 8B). 4-AP shifted the concentration-response curve for LEVO to the right without a change of the maximal relaxation (Emax without 4-AP: 96.4±6.7%, Emax with 4-AP: 93.4±8.2%, n=7). The potency of the inodilator was significantly decreased by 4-AP on the basis of the EC_{50} values (EC_{50} without 4-AP: 0.17±0.03 μM, EC_{50} with 4-AP: 0.30±0.02 μM, $p<0.01$, n=7).

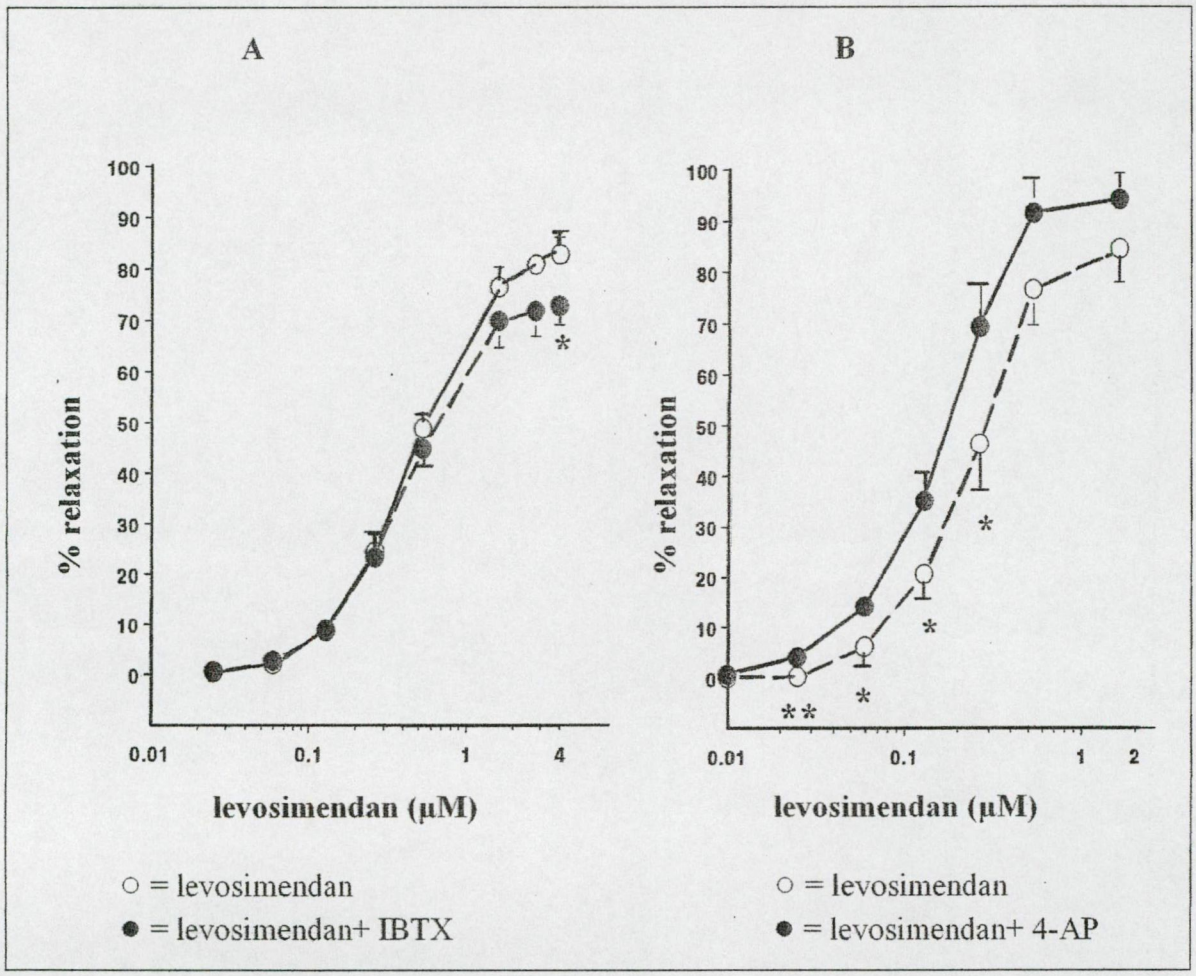

Figure 8. Effect of LEVO on the coronary artery tone in the absence (○) and presence (●) of the calcium-activated potassium channel blocker, 100 nM IBTX (A), or that of the voltage-dependent potassium channel blocker, 0.5 mM 4-AP (B). Coronary artery rings were precontracted with 20 mM KCl. Data represent mean±s.e.m. of 8 (A) and 7 (B) independent experiments. *$P<$ 0.05, **$P<$ 0.01 compared with the values obtained with the coronary artery preparations in the presence of a potassium channel blocker.

These experiments were repeated with a larger concentration of 4-AP, as depicted in Figure 9. 5 mM 4-AP resulted in a small but not significant enhancement of the KCl-induced tone (control: 65.7±8.7 mN, 4-AP: 73.1±6.8 mN, n=8), and it decreased considerably the maximum relaxing effect of LEVO (Emax without 4-AP: 101.8±6.6%, Emax with 4-AP: 68.2±6.7%, $P<0.01$, n=8) with no significant change in the potency value of the inodilator drug (EC_{50} without 4-AP: 0.19±0.03 μM, EC_{50} with 4-AP: 0.21±0.02 μM, n=8).

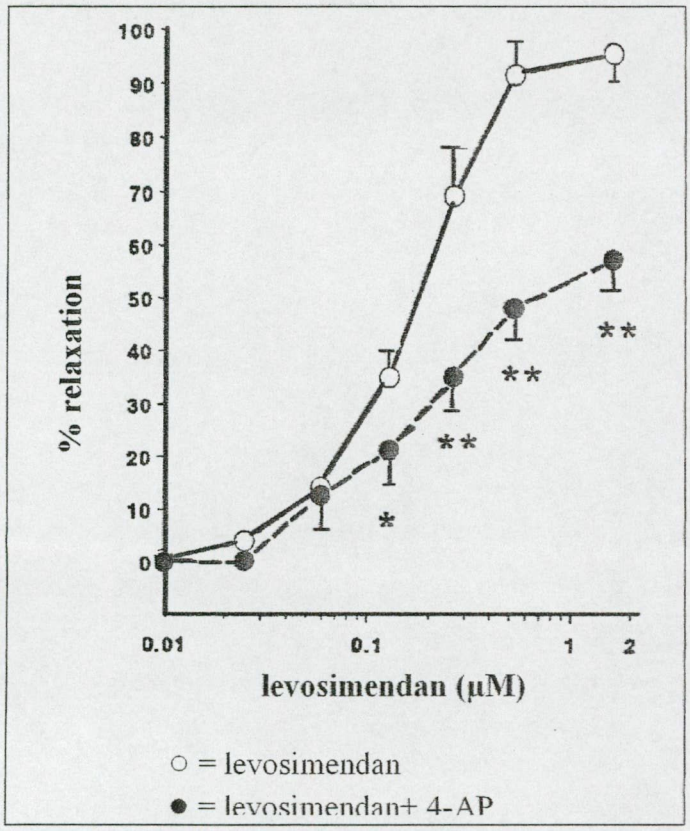

Figure 9. Effect of 5 mM 4-AP on the LEVO-induced porcine coronary artery relaxation. Concentration response curves represent the effect of LEVO on 20 mM KCl-induced contraction in the absence (○) and presence (●) of the voltage-dependent potassium channel blocker. Coronary artery rings were precontracted with 20 mM KCl. Data represent mean±s.e.m. of 8 independent experiments. *P<0.05, **P<0.01 compared with the values obtained with the coronary artery preparations in the presence of 4-AP.

3.3.2. Effect of levosimendan on human internal mammary artery

1 μM NA dose dependently contracted the isolated internal mammary artery (IMA) rings and the effect did not change significantly at various prestretched values (4.34±2.32 mN at 23 mmHg, 3.32±1.46 mN at 46 mmHg and 4.85±1.43 mN at 92 mmHg, n=16, ns). This suggests that there is no difference between the spasm reactions caused by sympathetic activity, in vitro. LEVO caused significantly higher relaxation at lower tones than at 92 mmHg. The vasorelaxing effect showed decreased tendency at increased pressure (0.6μM LEVO: -80±15.3% at 23 mmHg, -65.8±12.1% at 46 mmHg, -32.0±8.1% at 92 mmHg, n=8, P<0.05 23 vs 46 mmHg, Fig 10.).

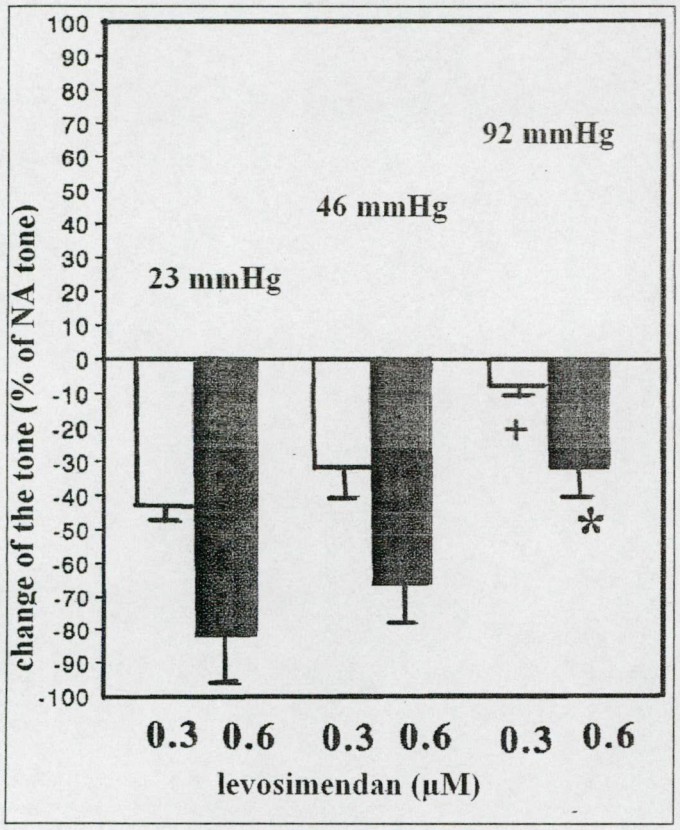

Figure 10. Effect of LEVO on Na-precontracted isolated human IMA at various simulated transmural pressure (23, 46, 92 mmHg). Values mean increase (+) and decrease (-) of the tone, where 100% is the maximum amplitude of NA contraction. IMA rings were precontracted with 1 μM NA. Data represent mean ± s.e.m. of 8 independent experiments. $^{+}P< 0.05$, $^{*}P<0.05$ compared with the similar concentration of LEVO obtained with the 23 and 46 mmHg pressure prestretching.

3.3.3. Effect of levosimendan in canine saphenous vein

Two different levels of calculated venous pressure (10 and 20 mmHg) were simulated in two parallel in vitro baths, separately. After NA induced contraction, LEVO caused a concentration dependent decrease of venous tone both at 10 and 20 mmHg (Fig. 11.). When basal tone was set at 20 mmHg, LEVO exerted significantly higher relaxations than at 10 mmHg. 3.4 μM LEVO caused 73.6±1.0% decrease of NA induced tone at 10 mmHg, (Fig. 11.). The venodilating effect was diminished by 1.5 μM GLI to 54.0±2.5%, $p<0.01$. At 10 mmHg venous pressure the 50% effective concentration (EC_{50}) of LEVO was calculated to be 0.75 μM in the presence of the drug alone and 1.1 μM in combination with GLI. In the simulated high intraluminal pressure group LEVO exerted a maximum of 91.4±1.0% decrease of NA-induced tone which decreased to 57.4±2.3% in the presence of GLI (EC_{50}: 0.96 μM LEVO alone and 1.26 μM LEVO+GLI, Fig. 13.).

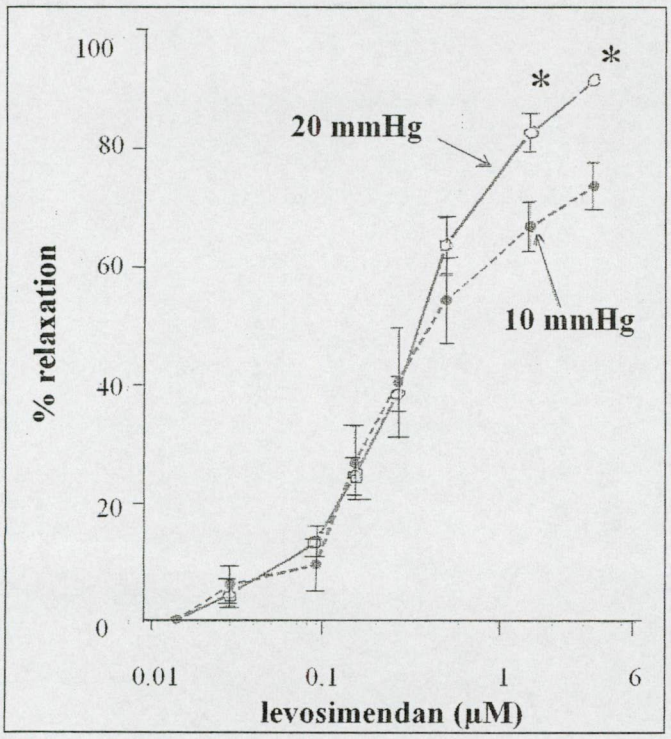

Figure 11. Effect of LEVO at enhanced levels of basal tone in canine saphenous vein. Concentration response curves represent the effect of LEVO at various prestretched tone. Venous rings were precontracted with 3.25 μM NA. Data represent mean±s.e.m. of 5 independent experiments. **P*<0.05 compared with the similar concentration of LEVO obtained with the 10 and 20 mmHg pressure prestretching.

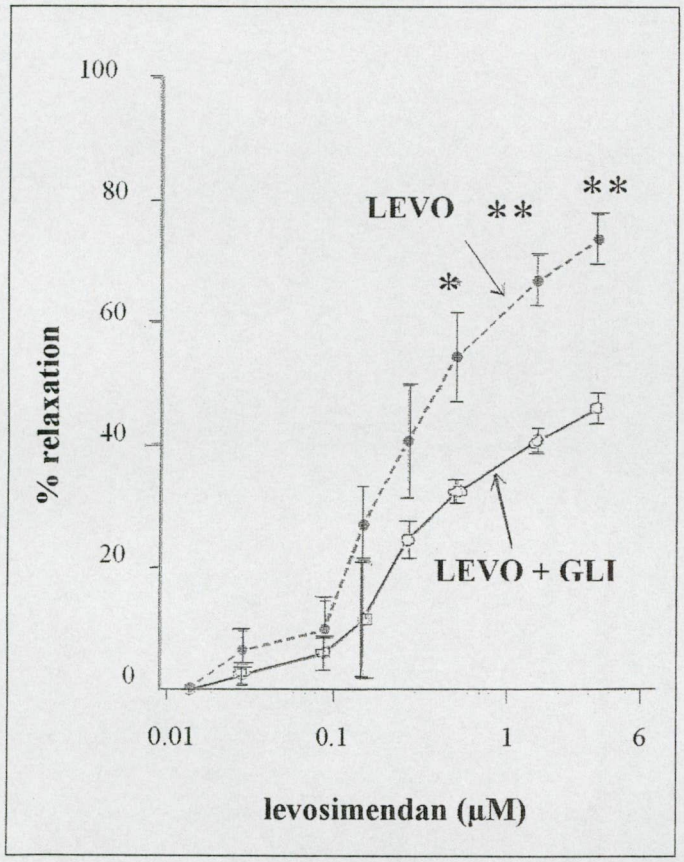

Figure 12. Effect of 1.5 μM GLI on LEVO-induced venodilation at 10 mmHg in canine saphenous vein. Concentration response curves represent the effect of LEVO at 10 mmHg basal tone in the presence (○) and absence (●) of 1.5μM GLI in NA precontracted (3.25 μM) veins. Magnitude of relaxations was expressed as percent of NA-induced tone. Data represent mean±s.e.m. of 5 independent experiments. **P*<0.05, ***P*<0.01 compared with the corresponding values of GLI treated saphenous vein preparations.

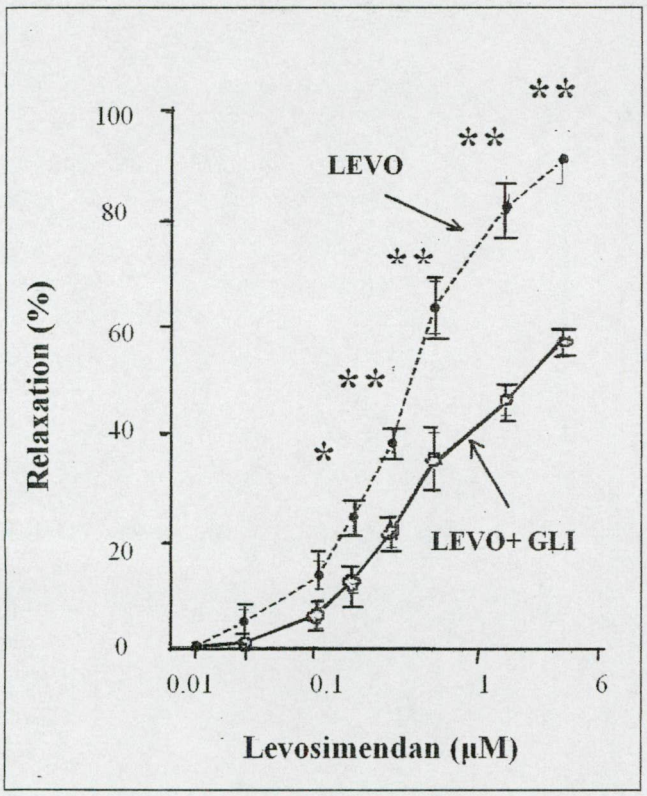

Figure 13. Effect of 1.5 μM GLI on LEVO-induced venodilation at 20 mmHg in canine saphenous vein. Concentration response curves represent the effect of LEVO at 20 mmHg basal tone in the presence (○) and absence (●) of 1.5μM GLI in NA precontracted (3.25 μM) veins. Magnitude of relaxations was expressed as percent of NA-induced tone. Data represent mean±s.e.m. of 5 independent experiments. **P*<0.05, ***P*<0.01 compared with the corresponding values of GLI treated saphenous vein preparations.

It can be seen that the GLI-sensitive component of LEVO-induced relaxation remained virtually the same at the two different levels of calculated venous tone. In all of our venous preparations EC_{50} of LEVO was below 1 μM.

3.3.4. Effect of LEVO in human portal vein

3.3.4.1. Effect of CRO and LEVO on the tone of human isolated portal vein

Both CRO and LEVO relaxed the NA-precontracted portal vein preparations almost completely (Fig. 14.).

Typical concentration-response registration were derived from one out of seven independent experiments in the case of both CRO and LEVO (Fig. 14A and B, respectively). On the basis of the EC_{50} values, LEVO was 16.1-fold more potent (EC_{50}: 0.281±0.03 μM, n=7) than CRO (EC50: 4.53±0.12 μM, n=7) under identical experimental conditions.

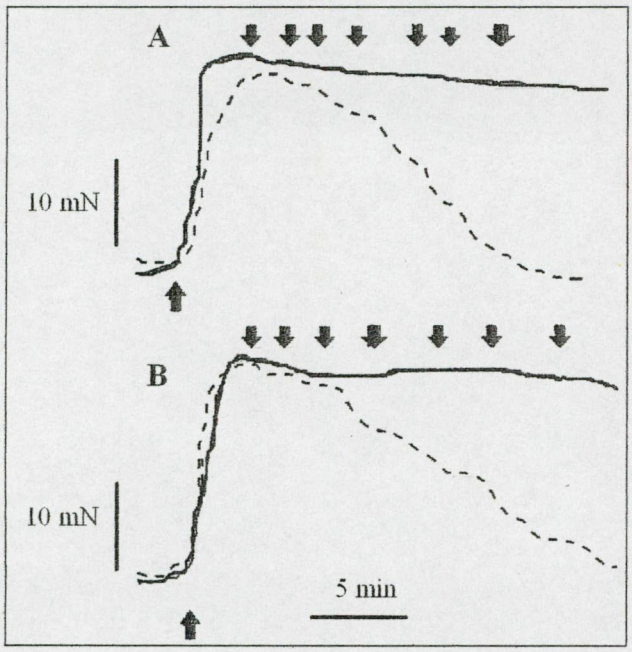

Figure 14. Typical concentration-response tracings for the relaxations induced by CRO (A) and LEVO (B) in human isolated portal vein. Contractions of venous rings were induced by 10 μM NA (upward arrows). Downward arrows represent the addition of cumulative concentrations of CRO (dotted line A: 0.2, 0.9, 2.5, 5.7, 12.0, 24.2 and 47.2 μM, respectively) and LEVO (dotted line B: 0.01, 0.03, 0.07, 0.15, 0.31, 0.63 and 1.3 μM, respectively) or the corresponding volumes of solvent (solid lines A and B). The original registrations are representatives of 7 independent experiments for each curve.

3.3.4.2. Effect of GLI on the venodilating action of CRO and levosimendan

Another series of experiments was aimed at determining the capacity of GLI to modify the relaxations induced by CRO and LEVO (Fig. 15.). Pretreatment of the portal vein preparations with 1.5 μM GLI for 30 min caused a significant leftward shift in the CRO concentration-response curve, but had no effect on the maximum relaxation response (Fig. 15A.) However, a ten-times-higher GLI concentration (15 μM) inhibited the relaxation by CRO (up to 48 μM) almost completely (Fig. 15A.). A low concentration of GLI (1.5 μM) also decreased LEVO-induced relaxation, the maximum amplitude of the relaxation was also depressed (Fig. 15B.). A high concentration of GLI (15 μM) further decreased the effect of LEVO, but this effect did not differ significantly from that produced by the low GLI concentration (Fig. 15B.).

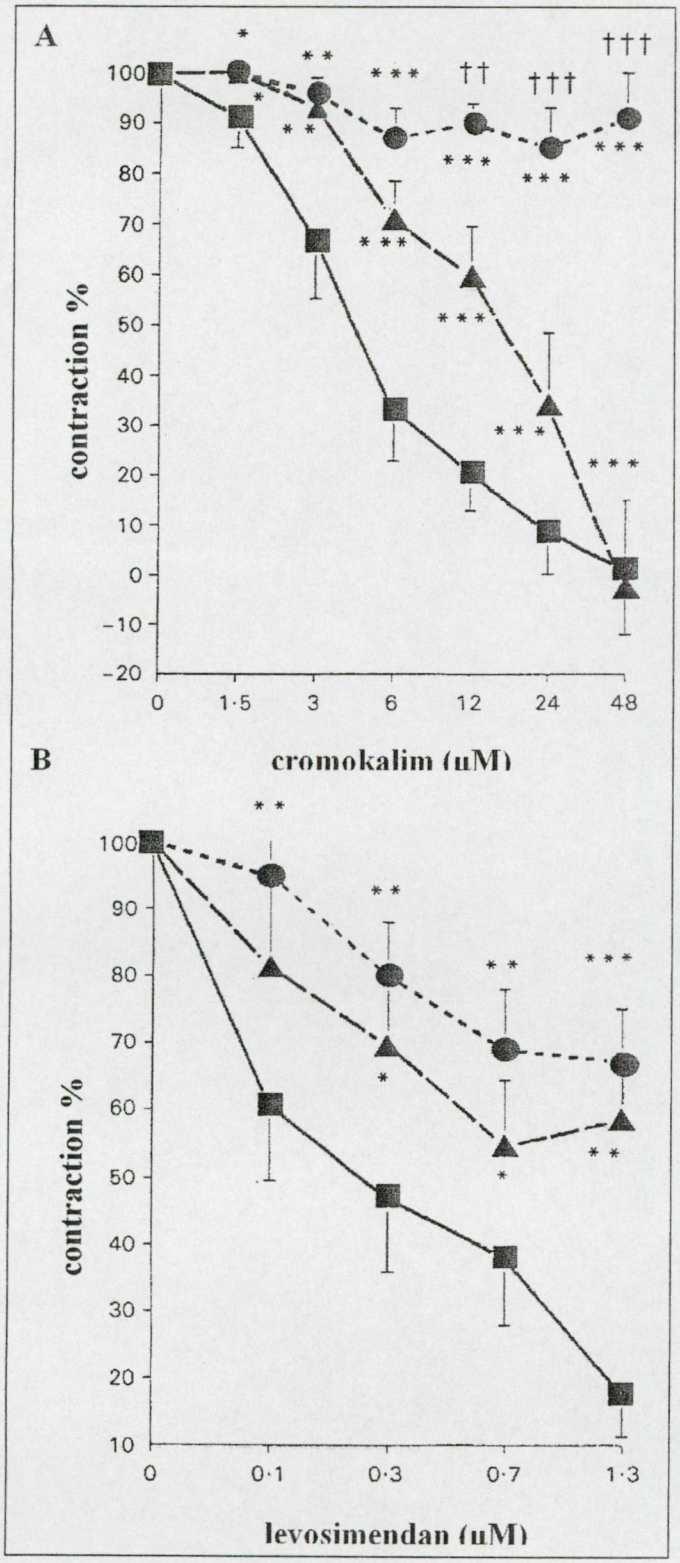

Figure 15. Effect of 1.5 μM ▲ and 15 μM ● GLI on the relaxations induced by CRO (A, ■) and LEVO (B, ■) in NA-precontracted human portal vein. Magnitude of contractions was expressed as percent of NA-induced tone. Data represent mean values ±s.e.m. of 7 independent experiments in the case of CRO and 6 in the case of LEVO. *$P<$ 0.05, **$P<$ 0.01, ***$P<$ 0.001 compared with CRO or LEVO alone; †† $P<$ 0.01, ††† $P<$ 0.001 compared with 1.5 μM GLI treatment.

3.3.4.3. Effect of GLI on the basal tone and on the NA-induced contraction

The basal tone, which was set up to 40 mN (about 4g), remained unchanged during the entire equilibration period (45 min). Even the large concentration of GLI (15 μM) applied did not influence this tone until the end of the 30 min incubation period (change in basal tone:

0.13±0.01 mN, n=7). Similarly, NA-induced contractions were not significantly affected by 15 μM GLI (17.3±1.6 mN vs 17.4±2.6 mN in the presence and absence of GLI, respectively, n=7).

3.3.5. Effect of levosimendan in human saphenous vein

3.3.5.1. Effect of GLI on the venodilating action of levosimendan

Relaxation by LEVO was studied in the presence and absence of GLI (Fig. 16A.). In 6 independent experiments, 30 min preincubation of the saphenous rings with GLI did not significantly influence the maximum amplitude of the 5-HT-induced tone (control: +12.70±6.36 mN vs GLI: +9.24±5.18 mN). LEVO produced a maximum of 28.1±7.5% relaxation with an EC_{50} of 0.32±0.04 μM. GLI abolished the relaxing effect of 0.13 and 0.31 μM concentrations of LEVO with a 40 times increase of the EC_{50} of the inodilator drug (12.8±0.8 μM).

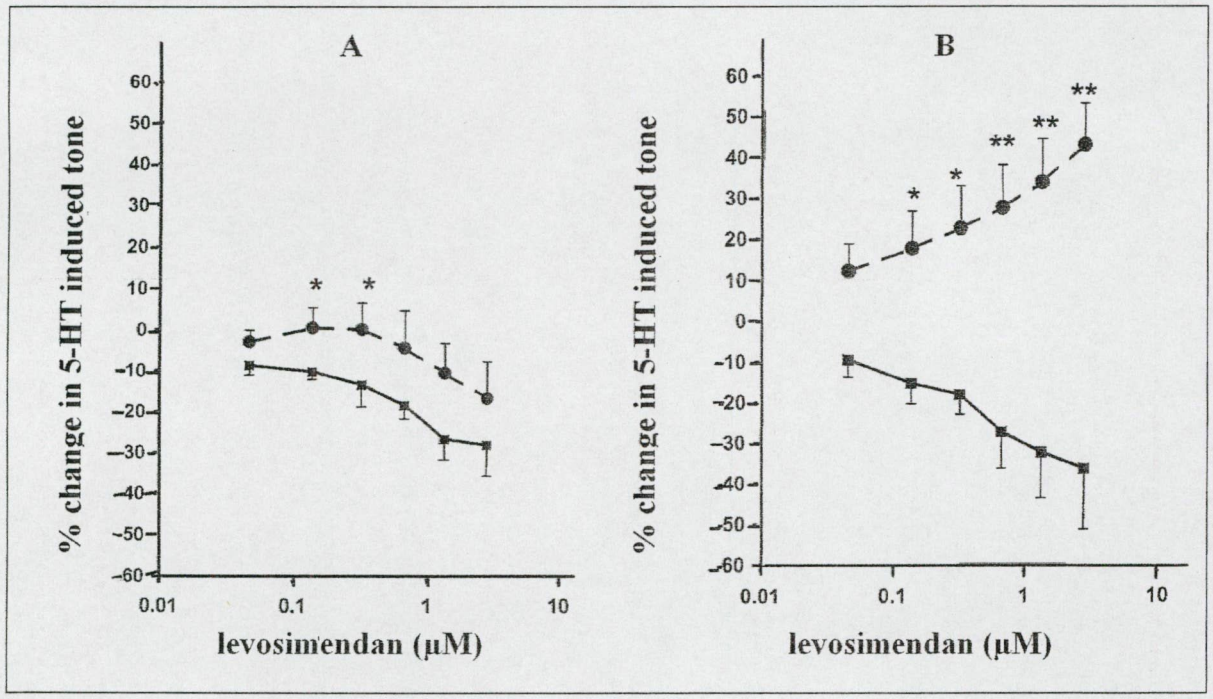

Figure 16. Effect of GLI and IBTX on LEVO-induced relaxation of human isolated saphenous vein. Part A: Effect of LEVO in the absence (■) and presence (●) of 15 μM GLI. Part B: effect of LEVO in the absence (■) and presence (●) of 90 nM IBTX. Values are expressed as percentage of the steady-state contraction induced by 5-HT and represent means ±s.e.m. of 6 experiments. Statistically significant difference from the effect of LEVO in the absence of GLI or IBTX: $*P< 0.05$, $**P< 0.01$.

3.3.5.2. Effect of IBTX on the venodilating action of levosimendan

In a separate series of 6 experiments, the effect of LEVO was investigated in IBTX-pretreated saphenous rings (Fig. 16B.). 30 min preincubation of the preparations with IBTX did not influence the 5-HT-induced tone (control: +24.73±17.11 mN vs IBTX: +20.53±10.42 mN). In these experiments, the maximum relaxation induced by LEVO was 36.5±14.9% with an EC_{50} of 0.25±0.03 μM. In the presence of IBTX, LEVO induced concentration-dependent contraction of the veins which became significant at and above 0.31 μM. For this effect the EC_{50} of the drug amounted to 0.21±0.06 μM.

4. DISCUSSION

4.1. Investigations on BK_{Ca} channel

4.1.1. Role of BK_{Ca} channels in the vasodilating mechanism of NO in canine coronary artery

Nitric oxide is released from the vascular endothelium of blood vessels and functions as a coronary artery autoregulator. The effector signalling of NO is known to be cyclic GMP in the smooth muscle cells that decreases the tone of arteries and veins. However, a hyperpolarizing mechanism of NO on the arterial smooth muscle membrane has also been suggested (Tare et al 1990). The sensitivity of vasorelaxation to the known hyperpolarizing potassium channel activator, CRO, and to NO as well as that of the specific antagonists of potassium channels, K_{ATP} and BK_{Ca}, revealed that NO is a potassium channel activator in canine conduit type of coronary arteries. The results also show that the K_{ATP} and BK_{Ca} ion channels are functionally distinct entities.

In canine coronary vessels, the K_{ATP} blocker, GLI inhibited the relaxation response to CRO, while IBTX, the known most selective BK_{Ca} blocker (Garcia et al 1991), decreased the relaxing effect of NO. Furthermore, the relaxation by CRO was not affected by IBTX at all and CRO is known to have no influence on cyclic nucleotides (Taylor et al 1988). A large density of BK_{Ca} channel was measured in the isolated smooth muscle cells of canine coronary arteries (Wilde & Lee 1989; Taniguchi et al 1993) and NO has been found to open BK_{Ca} channels in rabbit aorta (Bolotina et al 1994). These findings strongly support a functional connection between the endothelial mediator, NO and the effector BK_{Ca} channel at the level of the smooth muscle cells. In our experiments, the effect of 100 and 200 nM but not 400 and 800 nM NO was decreased by IBTX in a medium of low potassium (<20.4 mM KCl). Elevation of potassium concentration to 35.4-40.4 mM significantly depressed the relaxation by NO, and under this condition the IBTX-sensitive component disappeared. These results point to the important role of the membrane potential in the vasorelaxing mechanism of NO similar to the original observation in rat uterine artery (Tare et al 1990). Taken together all these findings we suggest that BK_{Ca} channel is also an effector signal and mediates, at least in part, the coronary autoregulatory function of the endothelial NO.

It is important to note that limited observations are available for a functionally distinct BK_{Ca} channel in the circulatory tree. In conscious dogs coronary dilating mechanisms by another K_{ATP} opener, pinacidil, and by sodium nitroprusside, a cGMP-elevating drug, can be separated by pharmacological means (Duncker et al 1993). However, using other blood

vessels, some observations support (Strong et al 1989; Green et al 1991), while others contradict (Gelband et al 1989; Okabe et al 1990) the findings in coronary arteries raising the possibility that the functions of K_{ATP} and BK_{Ca} channels can not be separated in some blood vessels. One possible explanation is that the prototype K_{ATP} opener, CRO, also relaxes smooth muscles through activation of BK_{Ca} channels, indicating a non-specific potassium channel activating charactheristics of this group of drugs (Balwierczak et al 1995).

Further evidences for functionally distinct potassium channels were obtained regarding the basal tone of canine isolated coronary arteries. Basal tone in our in vitro organ bath is represented by a passive stretch (10-40 mN) applied on a vascular ring perpendicular to the vessel wall. The basal tone was not affected by GLI but was increased by IBTX supporting a previous assumption that BK_{Ca} channels regulate the basal tension in epicardial coronary arteries of the dog, at least under in vitro conditions (Taniguchi et al 1993). Although K_{ATP} channels were also suggested to be an important metabolic autoregulator in canine coronary blood vessels at rest, this type of potassium channels appears to be localized in the smooth muscle of resistance coronary arteries (Ishibashi et al 1998).

Our present experimental results may have therapeutic relevance. NO is an active metabolite of nitroglycerine (NTG) and is released enzimatically from NTG in coronary arteries (Chung & Fung 1993). NTG increases cyclic guanosine 3,5-monophosphate (cGMP) in the smooth-muscle cell which is associated with relaxation (Ignarro & Kadowitz 1985). In isolated canine coronary arteries, the open probability of BK_{Ca} channels was increased by NTG, 8-bromo-cGMP (Fujino et al 1991) as well as by cGMP-dependent protein-kinase (Taniguchi et al 1993) suggesting the regulation of BK_{Ca} channels by the cGMP-messenger system. A functional effect of NTG, as a major coronary dilator, on BK_{Ca} channels has been demonstrated (Pataricza et al 1995). These findings are in agreement with the observation that 1 μM NTG decreased the amplitude of the action potential evoked by TEA (Harder et al 1979), a nonselective inhibitor of K_{Ca} channels (Nelson et al 1990).

In conclusion, there are at least two potassium channels, K_{ATP} and BK_{Ca}, that can be activated or inhibited in epicardial coronary arteries of dogs. We have demonstrated that exogenous NO has an IBTX-sensitive mechanism in its acute coronary artery vasodilator action in vitro.

4.1.2. Role of BK_{Ca} channels in the vasodilating mechanism of NO in human saphenous vein

NO caused dose-dependent relaxation in the IBTX pretreated and control saphenous vein rings and the relaxation was significantly smaller in the IBTX pretreated group. We have

demonstrated, for the first time, that BK_{Ca} channels regulate the tone of human saphenous vein. In the light of the above finding we suggest that the known decreasing effect of NTG in preload in vivo may also be due to NO-induced opening of BK_{Ca} channels (Hőhn et al 1995), a mechanism that is suggested to be functional in the epicardial coronary artery of the dog (Pataricza et al 1995).

In conclusion, NO has a vasodilator mechanism that differs from the known increase of cGMP in the smooth muscle cells both in the conduit coronary arteries and in the capacitive vein. This mechanism involves BK_{Ca} channel mediating a part of the relaxing effect of NO. We suppose that NO indirectly opposes the calcium entry into the smooth muscles of the coronary artery and saphenous vein through hyperpolarization of the cell mebrane. In a pharmacological point of view NO-donor drugs, such as NTG, may protect the coronary arterial and venous smooth muscle against depolarization-induced calcium overload (Brayden & Nelson 1992).

4.2. Investigations on K_V channel

4.2.1. Regulatory role of K_V channel in a human isolated coronary artery

4-AP, the known K_V channel blocking reference substance, concentration dependently enhanced the basal tone of a human coronary arterial ring. NIS, a blocker of L-type voltage-dependent potassium channels (L-VOCCs), partially reversed the 4-AP induced increase of the coronary arterial tone. This phenomenon corresponds to the hypothesis that depolarization enhances the open probability of L-VOCCs (Brayden & Nelson 1992) and the increase of calcium entry through the cell membrane is the mechanism by which 4-AP causes contraction in conduit coronary arteries.

4.2.2. Regulatory role of K_V channel on the basal tone of canine saphenous veins

Release of NA from the sympathetic nerve terminals has been shown to be regulated by K_V channels (Takata et al 1992). Venus blood vessels are richly innervated with sympathetic nerves, and potassium channels of K_V type appear to be present in some veins (Kato and Takata 1987; Hara et al 1980). In our experiments the K_V channel blocker, 4-AP was able to enhance the basal tone of isolated canine saphenous vein in a submicromolar concentration range, in vitro. The calculated EC_{50} value of the drug was 0.61 μM in the endothelium denuded venous rings only when the venous neuronal stores were filled with NA. This potency value is in the same magnitude of 4-AP concentrations measured in plasma

of patients suffering from spinal cord injury (27.7 ng/ml, i.e., 0.3 μM; Segal et al 2000) and almost the same as the serum level measured in multiple sclerosis (0.65 μM, Van Diemen et al 1993). 0.6-5 μM 4-AP was able to release NA from frog sympathetic ganglion (Kumamoto & Kuba 1985).

Chemical denervation by 6-hydroxydopamine (6-OHDA) of our saphenous preparations after filling (loading) the NA store of the venous tissue was performed in vitro. The hyperpolarizing potassium channels supposedly have an important role in the regulation of transmitter release, which was proved in animals of lower species (Kumamoto & Kuba 1985). Szentiványi et al proved the contractile effect of 4-AP on human capacitive vessels, but there was no evidence for the mechanism (Szentiványi et al 1997). The chemical damage with 6-OHDA of the sympathetic nerve terminals is an accepted method in vitro and also in vivo experiments (Huang et al 1995; Villanueva et al 1994; Medhurst et al 1993). 4-AP inhibited the voltage-dependent potassium channel, in milimolar concentration in smooth muscle cell investigations (Halliday et al 1995). However, in our studies 4-AP enhanced the basal tone in micromolar concentration, at least after filling the neuronal store with NA and this effect was completely inhibited with 6-OHDA. This finding supports an indirect neuronal mediation of the effect of low concentrations of 4-AP on venous tone.

Our conclusion is that the activity of K_V –type potassium channels largely determines the tone of capacitance saphenous vein through controlling the release of the sympathetic neurotransmitter, NA. Although epicardial coronary arteries also have autonomic innervation we did not measure the possible role of K_V channels in arterial neurotransmission. In porcine coronary artery, a work from Kun et al demonstrated that the contractile effect of 4-AP is partially mediated by neuronal 5-HT (Kun et al 2000). Therefore, in both capacitance veins and partly in conduit arteries, neuronal K_V channels regulate the vascular tension and this mechanism may be relevant for in vivo conditions when the sympathetic tone is elevated.

4.3. Investigations on the effect of the inodilator, levosimendan

4.3.1. Role of K_{ATP}, K_{Ca} and K_V types of potassium channels in the coronary artery dilating action of CRO and levosimendan in porcine coronary arteries

LEVO is an inodilator drug used to treat heart failure. In 1997, Yokoshiki et al demonstrated that the vasodilating action of LEVO is mediated through hyperpolarization of the smooth muscle membrane of isolated mesenteric arterial myocytes (Yokoshiki et al 1997a). The authors suggested that the hyperpolarizing effect was due to opening of K_{ATP}

channels by the inodilator drug. The K_{ATP} channel has also been proposed to be a target for LEVO in small coronary arteries of the dog (Kersten et al 2000) and in the human portal vein (Pataricza et al 2000). Our investigations indicate the activation of two other hyperpolarizing potassium channels, K_{Ca} and K_V, by LEVO in the epicardial coronary artery preparations isolated from the porcine heart. In our studies, four inhibitors of potassium channels were used to characterize the mechanism of LEVO-induced coronary artery relaxation.

The interaction between CRO and GLI revealed the presence of functional K_{ATP} channels in our isolated porcine coronary artery preparations. The same concentration of GLI, however, was without any effect on the relaxation induced by LEVO. The opposite results were obtained with 2 mM TEA, a non-specific blocker of K_{Ca} channels: TEA had no effect on CRO whereas it decreased the maximum effect of LEVO. This finding strongly supports the role of different subtypes of potassium channels in the vasorelaxing mechanisms of CRO and LEVO. IBTX, the BK_{Ca} blocker, caused a small but not significant decrease in the maximum effect of LEVO suggesting the involvement of the large-conductance subtype of K_{Ca} channels in the vasodilating mechanism. The effect of the inodilator was also significantly decreased by 0.5 mM 4-AP, a potent inhibitor of the K_V channels in this type of blood vessels (Shimizu et al 2000). Interaction with 4-AP was evident even at as low as 0.05 μM concentration of the inodilator drug. Thus, the low submicromolar concentrations of LEVO seem to activate K_V channels, while the drug, in larger concentrations, activates both K_V and K_{Ca} channels in porcine epicardial coronary artery. Ten-fold larger concentration of 4-AP (5 mM) decreased the maximum effect of LEVO by 43%. However, this concentration of 4-AP is non-specific and has also been shown to affect the BK_{Ca} channels in this artery (Fujino et al 1991; Petkova-Kirova et al 2000). Therefore, the functional effect of two potassium channels, the K_V and K_{Ca} channels, are presented as targets for the effect of the inodilator, LEVO, in the porcine coronary artery .

One might argue for the lack of effect of the LEVO on the K_{ATP} channels, a known target for the drug in other vascular tissues (Yokoshiki et al 1997a; Pataricza et al 2000; Kersten et al 2000). The functional importance of this type of potassium channels is evident in the present investigation. It is important to note that some pharmacological and electrophysiological properties of K_{ATP} channels in porcine coronary artery have been suggested to differ from those of other blood vessels (Inoue et al 1989; Wakatsuki et al 1992). The non-specific potassium channel activating property of LEVO observed in the present experiments is also supported by the agonist activity of the drug on mitochondrial K_{ATP}

channel (Kopustinskiene et al 2001) which is pharmacologically different from those K_{ATP} channels located in the cell membranes.

LEVO is an approved drug under the trade name of Simdax for treating severe heart failure (Papp et al 1999). Another important finding of the present work is that the drug-induced decrease of the large epicardial coronary tone could be achieved at therapeutically meaningful concentrations (Sandell et al 1995). This effect of the inodilator may also be relevant to the human coronary arteries in vivo (Krassói et al 2000). Furthermore, the interaction of LEVO with 4-AP might have clinical significance because 4-AP is a therapeutic tool in some neurologic disorders (fampridine).

In the light of the present findings we conclude that LEVO effectively and directly decreases the tone of the epicardial coronary artery preparations isolated from the porcine heart. This effect may induce coronary dilation in vivo in those submicromolar concentrations of the drug which were proved to exhibit inotropic effect on the heart (Haikala & Lindén 1995). It appears that the mechanism of vasodilating action of LEVO depends, at least in part, on potassium channels functionally differing from the K_{ATP} channels.

It is important to mention that a species difference exists concerning the regulation of the basal coronary artery tone by BK_{Ca} channels. Under in vitro conditions, canine but not porcine coronary artery showed an elevation of the resting tone after administration of IBTX. While K_V channels appear to have regulatory role in the basal tone of human coronary artery, evidence for the role of BK_{Ca} channels is lacking at present.

4.3.2. Vasodilation by levosimendan in relation to simulated transmural pressure in human internal mammary artery

During bypass surgery hypoxia, low temperature and low perfusion pressure can provoke spasm of bypass conduits including IMA. We simulated different perfusion pressures in isolated IMA rings obtained from coronary artery bypass surgery. Passive stretches were applied perpendicular to the wall of IMA under in vitro conditions and calculated as 23, 46 and 92 mmHg transmural pressures according to the Laplace law.

NA-induced contractile responses did not differ at the three simulated values of pressure suggesting that the elevated sympathetic tone alone might contribute to contraction independently of the actual transmural pressure of the IMA graft.

Myogenic tone of the blood vessels is activated when the transmural pressure is high according to the Starling law. The vasodilating effect of 0.3 and 0.6 μM LEVO decreased with increasing pressure of IMA, indicating that depolarization associated with enhanced

myogenic tone counteracts the effect of a hyperpolarizing vasodilator. Although we did not present evidences that LEVO hyperpolarized the smooth muscle of this conduit graft, the inodilator drug was shown to activate K_{ATP} channels in isolated IMA samples (Yildiz et al, personal communication). Under identical conditions, nifedipine exerted maximum vasodilation at a medium level of pressure (46 mmHg) supporting the maximum activation of calcium channels at higher transmural pressure and consequently more depolarized state of IMA in comparison to the activation of potassium channels (Hegedűs et al 2002).

In conclusion LEVO is an effective dilator of isolated IMA in submicromolar concentrations and may have important therapeutic value especially at low perfusion of the graft in the perioperative setting.

The limitation of the above method is that the Laplace law serves only approximate pressure values because the equation can be applied for those small blood vessels in that the wall thickness/diameter ratio is smaller than about 1/100.

4.3.3. Vasodilating effect of LEVO at different levels of simulated pressure in canine saphenous vein

Veins from the saphenous region are used extensively to test vasodilator substances because they are highly selective to the effects of cardiovascular factors (Hollander et al 1976). Canine saphenous veins were found to possess K_{ATP} channels (Nakashima & Vanhoutte 1994) and responded with changes in tone to the effect of different potassium channel activators and inhibitors. In this section of the work, submicromolar concentrations of LEVO have shown to exert similar effectiveness at moderate (10 mmHg) and high (20 mmHg) venous tones simulated by the enhancement of perpendicular wall stretch under in vitro conditions. 1.5 μM GLI significantly inhibited LEVO-induced relaxations even in the submicromolar concentration range of the inodilator drug. This was evident at the two different values of pressure. These results are in accordance with our observations obtained in saphenous and human portal vein (Pataricza et al 1998). In the present study, we did not use higher concentrations of the K_{ATP} channel inhibitor, GLI, because of its known non-specific action on other ion channels (e.g. chloride channel). 1.5 μM GLI represents a therapeutic concentration of the antidiabetic compound that can cause a decrease but not complete inhibition of LEVO-induced venodilation. The sensitivity of the effect of LEVO to inhibition by GLI is known from other studies including arterial myocytes (Yokoshiki et al 1997a) and cardiac ventricular cells (Yokoshiki et al 1997b). However, it appears that the venous smooth muscle is the most sensitive type of vascular tissues with respect to the activation of K_{ATP}

channels by LEVO. This mechanism may play a role in the therapeutic effect during administration of the drug to patients suffering from congestive heart failure.

In conclusion, LEVO is an effective venodilator at two simulated venous pressures. The inodilator appears to be effective at higher transmural pressure, an effect that may decrease pathologically elevated preload in heart failure. Under these conditions, LEVO retains its sensitivity to GLI and interaction of the two drugs may have important therapeutic consequences.

4.3.4. Role of K_{ATP} channel in the vasodilating effect of LEVO in human portal vein

The K_{ATP} channel activator, CRO relaxed the NA-contracted human isolated portal vein. LEVO, which was shown to increase the open probability of K_{ATP} channels in isolated arterial myocytes (Yokoshiki et al 1997a), was also found to be effective and to be a more potent relaxant than CRO in human portal venous preparations. GLI, a K_{ATP} channel blocker, inhibited the CRO-induced relaxation of the portal vein, while it partially decreased the effect of LEVO. GLI did not change the basal tone of portal veins and also did not significantly affect the contractions induced by NA. This finding supports the specificity of interactions between GLI and CRO or LEVO. However, it is worth mentioning, that the 15 μM GLI-sensitive component of the LEVO action might also be related to mechanisms other than opening K_{ATP} ion channels (Tominaga et al 1995).

An increase in sympathetic nervous activity has been recognized in cirrhosis and heart failure associated with portal hypertension or congestion. Portal vasoconstriction to NA was shown to be enhanced in experimental cirrhosis (Mathie et al 1996). In this part of our work, both CRO and LEVO antagonized the contractile effect of NA in the portal vein, although neither of the drugs have adrenergic-receptor-blocking activities (Harkin et al 1995). The possible significance of administering CRO and LEVO in portal hypertension would produce a selective effect on the portal circulation as compared with the effect of these drugs on the splanchnic arterial bed. We have no functional evidence for this assumption, but the low potency of LEVO for opening K_{ATP} channels in mesenteric arterial myocytes (EC_{50}= 2.9 μM; Yokoshiki et al 1997c) strongly suggests this possibility. Furthermore, it has been demonstrated that the tone of NA-contracted portal vasculature was sensitive to GLI (Mathie et al 1996), while the splanchnic circulation was not affected (Ralevic et al 1996). Our present experimental observations point to the role of a CRO-type vasodilator mechanism in the human portal vein which could be activated by drugs involving GLI-sensitive potassium channels.

The inodilator drug, LEVO, has been shown previously to decrease the preload of the heart in intact conscious dogs (Harkin et al 1995) and in dogs with failing heart (Udvary et al 1995). The maximum inotropic effect of LEVO was attained at submicromolar concentrations (0.2 μM; Sandell et al 1995; 0.3 μM, Haikala et al 1997), corresponding to the effective concentrations of the drug in human portal vein.

In conclusion LEVO might be beneficial as an adjuvant drug for treating portal hypertension.

4.3.5. Role of KATP and BKCa channels in the vasodilating effect of levosimendan in human saphenous vein

In this part of our work, LEVO was found to decrease the 5-HT-induced tone of human isolated saphenous vein. The drug caused venodilation in concentrations which are attained in the clinical therapy of heart failure. Our finding supports a previous assumption that the LEVO-induced diminution of cardiac preload is due to a direct action of the drug on the venous tone (Harkin et al 1995; Udvary et al 1995). The action in the human portal vein (chapter 4.3.4.) and in human saphenous vein suggest a receptor independent vasodilation induced by the drug as it was also found in isolated porcine coronary artery (Gruhn et al 1998).

Venodilating effect of LEVO was partially inhibited by GLI, reflecting some role of the hyperpolarizing ATP-sensitive potassium channels (K_{ATP}) in the mechanism of action of the drug. The K_{Ca} channel appears to be important in regulation of the saphenous tone (Hőhn et al 1995). Therefore, the involvement of this type of potassium channels was plausible (Patarícza et al 2003). Furthermore, the BK_{Ca} channel revealed cation activation characteristics similar to troponin C (Lattore et al 1989), the latter being absent in smooth muscles, but comprising the primary target site for LEVO in the heart (Haikala et al 1995). In the present study IBTX, a selective inhibitor of BK_{Ca} channels, interacted with the venodilating effect of LEVO. This type of interaction unmasked concentration-dependent contractile effect and was without a change in the EC_{50} value of the inodilator (Hőhn et al. 2004). This suggests the presence of different binding sites for LEVO and IBTX in the saphenous vein. The exact mechanism involved is, however, speculative for the time being. Nevertheless, such interaction at submicromolar concentrations of LEVO may offer a future therapeutic consequence of our finding.

The BK_{Ca} channel is a "blood vessel-specific" potassium channel which, according to our current knowledge, has no functional role in the heart. Thus, we suppose that any

pharmacological modulation of BK_{Ca} channels, which is a developing field in vascular research, interferes only with the vasodilating but not with the inotropic effect of LEVO (Pataricza et al 2003, Yokoshiki & Sperelakis 2003).

In conclusion, we have provided evidence for a so far unknown effect of LEVO in a human capacitance blood vessel, an interaction with a hyperpolarizing BK_{Ca} channel blocker. In addition to its known K_{ATP} channel activating property, a presumed effect of the inodilator drug on the BK_{Ca} channel might contribute to the decrease in cardiac preload. The consequence would be a beneficial haemodynamic effect complementing the inotropic action of LEVO in the treatment of severe heart failure.

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7. ANNEX

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS